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BY MARGARET G SMITH M.D., RUSSELL J BLATTNER, M.D., AND FLORENCE M HEYS PH.D

(*From the Department of Pathology and the Department of Pediatrics, Washington University School of Medicine St Louis*)

(Received for publication, April 8, 1946)

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Materials and Methods

Uninfected Mite Colonies—A homogeneous colony of uninfected chicken mites was established from a single adult female and her nymph offspring A single engorged female was removed from a parent colony shown by repeated tests to be free of the St. Louis encephalitis virus This single female mite was placed in a glass tube and observed for egg laying After the larvae had hatched and metamorphosis to first stage nymphs had occurred the mother and the nymphs were placed in an autoclaved container These mites were fed repeatedly on normal chickens which had hatched from eggs in the laboratory and which were protected at all times from possible outside infection. In this manner a large colony of homogeneous uninfected mites was established. From this colony subcolonies were set up when required for experimental purposes.

On a number of occasions these subcolonies were retested as follows for the possible presence of the St. Louis encephalitis virus, always with negative results. Mites collected from these colonies on Feb 4 1945 Apr 6 1945 Apr 11 1945 and two lots of mites collected on

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

ST LOUIS ENCEPHALITIS

Infection of Chicken Mites, Dermanyssus gallinae, by Feeding on Chickens with Viremia, Transovarian Passage of Virus into the Second Generation*

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used to establish the colony since the sample tested for virus 4 weeks later consisted only of first stage nymphs

A second colony of infected mites was established with freshly engorged mites, adults and nymphs, collected after feeding on chicken 4-2 shown to have viremia. This colony has been maintained until the present time (8 months) by feeding on normal chickens bred in the laboratory. Six months after the colony was established with infected mites the St. Louis virus was recovered from a sample consisting of 100 mites, adults and nymphs, collected from the colony.

Thus, the RN_6 strain of St Louis encephalitis virus was recovered from mites collected from 2 experimentally infected colonies, 4 weeks after establishment of the first colony and 6 months after establishment of the second colony.

Transovarian Passage through the Second Generation.—Further experiments demonstrated transovarian passage of the St Louis virus through the first and second generations of experimentally infected mites.

On June 2, 1945, chicken 5-8 was inoculated subcutaneously with the egg membrane strain of the St Louis virus, and viremia was demonstrated 52 hours after inoculation. Engorged adult mites fed on this chicken during viremia were collected on June 5, 1945, and each adult mite was placed in a separate glass tube to be observed for egg laying. The adult females were separated from the unhatched eggs. Some of the adult female mites were saved for the experiment described in the following section. On June 11, 1945, the remaining 130 adult females were triturated in tryptose phosphate broth and the suspension inoculated into 7 mice, 9 days of age. Four of the 7 mice showed signs of illness in 12 to 17 days. Mice inoculated intracerebrally with brain tissue from these 4 mice developed convulsions in 3 to 5 days.

Eggs separated from the above female mites were allowed to hatch and the larvae to metamorphose to first stage nymphs. These nymphs were divided into 2 lots. One lot of 153 nymphs was triturated in tryptose phosphate broth and the resulting suspension inoculated intraperitoneally into 7 mice, 9 days of age. Five of the 7 mice showed signs of illness in 12 to 14 days, and an infective agent was passed readily to adult mice by intracerebral inoculation. The virus thus obtained from the adult female mites and from their unfed first stage nymph offspring was identified as that of St. Louis encephalitis.

In order to demonstrate transmission of the virus through the egg into the second generation, the other lot of unfed first stage nymphs, several hundred in number, was used to establish a new colony. The colony was fed on 3 different normal chickens at 72 hour intervals, thus permitting the feeding of mites on a normal chicken as first stage nymphs, again as second stage nymphs, and finally as adults. After the third feeding, engorged adults (*i e.*, adults of the first generation since feeding on infected blood) were placed in individual tubes for egg laying. The adult females were separated from the unhatched eggs. These second generation eggs were allowed to hatch and the larvae metamorphose to first stage nymphs.

One hundred and twenty-five of the first generation adult mites were triturated in broth and the suspension injected intraperitoneally into 5 mice, 9 days of age. Three of the 5 mice developed signs of illness in 12 to 13 days. Intracerebral passage of each of the 3 brains to adult Swiss mice produced typical convulsions in 3 days. Likewise, 165 unfed first stage nymphs of the second generation *i e*, since feeding on infected blood, were triturated in broth and the suspension passed by intraperitoneal inoculation to 7 mice, 10 days of age. Three of the 7 showed signs of illness in 12 days, and brain tissue from each was passed to adult mice by intracerebral inoculation. As before, the virus was identified as that of St Louis encephalitis

Thus, transovarian passage of the St Louis virus into the second generation has been demonstrated in mites infected in the laboratory

Transovarian Passage, Persistence of Virus through Second Egg Laying Period —

The adult female mites reserved for this experiment were a part of those fed on chicken 5-8 and separated from their unhatched eggs. Other adult females of this collection and also their unfed first stage nymph offspring had yielded the St Louis encephalitis virus, as recorded in the preceding section. The adult female mites reserved for this experiment were placed in an autoclaved container and allowed to feed on a normal chicken. After feeding, these adults were collected and placed in individual glass tubes for further egg laying[2] and, as before, were separated from their unhatched eggs These females and their unfed first stage nymphs were tested separately for the presence of the St. Louis virus. Thirty two adults were triturated in tryptose phosphate broth and the suspension was inoculated intraperitoneally into 7 mice, 9 days of age. Two of the 7 showed signs of illness in 12 to 13 days and passage mice inoculated intracerebrally developed convulsions in 4 days. Likewise, 130 first stage nymph offspring of these females were triturated in broth and the resulting suspension inoculated intraperitoneally into 8 mice, 6 days of age Three of the 8 showed signs of illness in 12 to 13 days, and secondary intracerebral passage to adult mice produced convulsions within 4 days. The virus recovered from both the adult females and their nymph offspring was identified as that of St. Louis encephalitis.

These results show that a female mite, infected as an adult, can pass the St. Louis virus, not only through the eggs laid immediately after the infective feeding, but also through the eggs laid after an additional feeding on normal blood

DISCUSSION

Hammon, Reeves, and Gray (4) have presented data from field and laboratory investigations which support the hypothesis that both St. Louis encephalitis and Western equine encephalomyelitis are mosquito-borne, and that the source of the infection for the mosquito is an inapparent reservoir among vertebrates, particularly the domestic fowl. However, neither the virus of St. Louis encephalitis nor that of Western equine encephalomyelitis has been isolated from hibernating mosquitoes, nor is there evidence that these viruses persist in the blood of experimentally infected fowl for longer than a few days Accordingly, the mechanism by which these viruses persist from year to year in an endemic area has not been explained

Chicken mites (*Dermanyssus gallinae*) in several localities in St. Louis County have been shown to be infected with the virus of St. Louis encephalitis (1, 2), probably permanently, since congenital transfer of the virus in these mites has been demonstrated. Furthermore, the persistence of the virus in nature is indicated by the isolation of the virus from mites collected at a single site at intervals of 6, 8, and 16 weeks (2) The virus has persisted for months in 2

[2] The chicken mite lays several sets of eggs after a single mating However a blood meal is required before each set of eggs is laid

laboratory colonies, one established with mites found infected in nature (2), and one with experimentally infected mites. However, until it has been shown that infected mites are capable of transferring the virus of St. Louis encephalitis to chickens by bite, the epidemiological significance of the persistence of virus in mites remains conjectural.

Sulkin (5) has isolated the virus of Western equine encephalomyelitis from *Dermanyssus gallinae* collected on a ranch in Texas where this disease had occurred in horses. As in the case of St. Louis encephalitis, the epidemiological significance of this finding cannot be determined without further evidence.

SUMMARY

A colony of chicken mites (*Dermanyssus gallinae*) was established from a single adult female mite and her offspring. This colony of mites was shown to be free of the virus of St. Louis encephalitis. Infection of mites from this homogeneous colony with the virus of St. Louis encephalitis was accomplished by feeding on chickens having viremia. The virus was recovered as readily from mites which had not been allowed to feed for 8 days as from mites freshly engorged, showing that the demonstration of virus in the mites does not depend on the presence of fresh infective chicken blood. Transovarian passage of the St. Louis virus into the second generation has been demonstrated in mites infected experimentally. The female mite infected as an adult can pass the St. Louis virus through eggs laid after additional feeding on normal blood. Persistence of the virus for a period of 6 months has been shown in a colony of mites infected experimentally in the laboratory.

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STUDIES ON ENDEMIC PNEUMONIA OF THE ALBINO RAT

I. The Transmission of a Communicable Disease to Mice from Naturally Infected Rats

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(Received for publication, March 28 1946)

The natural occurrence of an inflammatory reaction in the lung of the albino rat is now a matter of common knowledge The condition being progressive with ultimate purulent cavitation is sometimes referred to as bronchiectasis. Since it is initially a bronchopneumonia it will be regarded as such in the present work and called endemic pneumonia of the albino rat. It might also be known as atypical or symptomless pneumonia as it is so well tolerated by the host that its certain recognition is often dependent on a direct examination of the lung It is known that the disease is universally distributed and that its development though unrelated to the diet of the host is correlated with its age, immature rats being significantly less susceptible than adult animals. Aside from these facts, however, there is little knowledge of its natural history

The rat itself has been one of the chief handicaps in the study of endemic pneumonia, the younger animals being relatively resistant and the older ones showing a high natural incidence of pulmonary involvement Moreover, the rat is subject to a second and distinct respiratory disease, called by us (1) infectious catarrh, which may also result in pneumonia although it more commonly affects the nasal passages and the middle ears This disease is likewise widely distributed and may coexist with endemic pneumonia.

Some years ago Nelson and Gowen (2) attempted to establish a disease free colony of albino rats by selective breeding The procedure was successful in eliminating infectious catarrh but failed to free the colony of endemic pneumonia It was noted, however that the rate and severity of the pulmonary reaction was appreciably reduced after selection. At that time (1931) there was no adequate explanation for this difference infectious catarrh not having been recognized as a distinct entity until later (1940) It now seems probable that this discrepancy before and after selection was due to the elimination of infectious catarrh which in the original colony had coexisted with endemic pneumonia.

In 1939 King (3) undertook to eliminate labyrinthitis, with its resultant disequilibration, from a colony of albino rats with a high natural rate of the disease Gray, captive female rats known to be rarely infected were used as foster mothers for albino rats removed from their mothers at or soon after birth Eventually a group of disease-free albino breeders was obtained and used as the nucleus of a colony

which subsequently remained essentially free of the disease With respect to endemic pneumonia, however, the results of Miss King's procedure were similar to ours Her method was successful in ridding the colony of labyrinthitis but failed to free it of endemic pneumonia

Since there was no entirely suitable natural host at hand for experimental purposes it seemed desirable to attempt the transmission of endemic pneumonia to some other host in which it did not occur as a natural disease We (4) had shown earlier that the white mouse was highly susceptible to infectious catarrh of the albino rat, and having at our disposal a mouse colony which had been notably free from any respiratory disease for many years, the mouse was chosen as an alternative host The work herewith reported is concerned with our endeavor to reproduce experimentally in the mouse the type of reaction which occurs naturally in the lung of the rat

Source and Nature of the Material Used in the Mouse Transmission Experiments

Unless otherwise indicated the material used in the following experiments was obtained from the selected colony of albino rats maintained at The Rockefeller Institute in Princeton The rats in this colony tend to develop pneumonia as they mature but are free from infectious catarrh Material from other rat colonies was generally unreliable because of the coexistence of endemic pneumonia and infectious catarrh One additional source of uncomplicated endemic pneumonia was furnished, however, by a group of albino rats obtained through the courtesy of Miss Gladys Sperling from a colony maintained at Cornell University in Ithaca, New York

The pneumonia rate originally reported for adult rats, upwards of 1 year in age, from the selected colony was 52 per cent as compared with 78 per cent in rats from another unselected colony (2) There was no pneumonia in young rats, 3 to 4 months old, from either colony None of the rats from the selected colony, in either age group, showed middle ear involvement. Beginning in 1940 when the colony was approximately 10 years old, 70 additional adult rats 9 to 14 months old were killed and examined The incidence of pneumonia in these rats was 62 8 per cent. During this period 100 young rats, 1 to 3 months old, were also examined and in contrast to the original findings 8 per cent showed pneumonia at autopsy

As noted earlier by Hektoen (5) and by Nelson and Gowen (6), there was a marked variation in the lung lesions from rat to rat and from lobe to lobe in the same rat In general two types of reaction were observed The first was characterized by partial or complete consolidation of one or more lobes with localized abscess-like areas containing exudate The second type showed small patchy or spherical foci of consolidation which contrasted sharply with the surrounding normal lung tissue when examined with a dissecting microscope Low power magnification was regularly employed in the subsequent examination of the lung from both rats and mice The two types of reaction were about equally divided, 21 of the 44 adult rats with pneumonic lungs showing the first type and 23 the second

Involvement of the middle ear is not a characteristic finding in endemic pneumonia as it is in infectious catarrh No cases of otitis were observed in the 50 adult rats originally examined from the selected colony In the second examination 3 of the 70 adult rats showed a

purulent exudate in the middle ears a rate of 4.2 per cent as contrasted with 72 per cent in the adults from the unselected colony Two of these cases were observed before mouse tests were used but microscopic examination of the exudates failed to show the characteristic coccobacilliform bodies of infectious catarrh The third was tested in mice with results that pointed to a direct relation with endemic pneumonia, indicating that otitis while rare may be an accompanying feature of the disease

Endemic pneumonia may also be accompanied by a frank rhinitis with a copious mucopurulent exudate present in the nasal passages but it is of rare occurrence in the selected rat colony A low-grade irritation of the nasal mucosa, particularly in adult rats is sometimes indicated by the microscopic presence of leucocytes without definite exudation. Snuffling may likewise be observed but is such a variable symptom that it is of doubtful diagnostic value. It occurs in young rats with no evidence of pneumonia at autopsy and may be absent in adult rats with advanced lesions in the lung

The Nasal Instillation of Mice with Pneumonic Lung Suspensions from Adult Albino Rats

The first experiment in which mice were injected intranasally with a suspension of pneumonic lung tissue from old albino rats resulted in an inflammatory irritation of the lung, middle ears, and nasal passages. The symptoms and macroscopic pathologic findings of the infected mice were then indistinguishable from those of infectious catarrh (7) and it was feared that this disease had reappeared in the previously free rat and mouse colonies Subsequent microscopic examination of these and other infected mice clearly indicated that this was not the case and that a disease entity distinct from infectious catarrh had been transmitted to the mice through the agency of the rat lung suspension

In the initial experiment 5 white mice, 15 to 18 gm. in weight were injected intranasally while under ether anesthesia with a lung suspension from two 12 months old albino rats. The suspension included both pneumonic and normal appearing lung tissue which was finely divided with a glass tissue grinder and diluted to a concentration of approximately 10 per cent with normal or buffered saline solution (pH 7 4) About 0 05 ml was instilled nasally in anesthetized mice by dropping the suspension from a syringe on the nares. A similar procedure was followed in all of the subsequent work.

Four weeks after injection 3 of the 5 mice, which then chattered intermittently were killed and at autopsy showed consolidation of one or more lobes of the lung together with a purulent otitis and a mucopurulent rhinitis. The 2 remaining mice, which also chattered, were used in a contact experiment but subsequently died and were not examined.

From time to time 13 additional transfers were made from diseased rats to mice All of these transfers were followed by a recognizable inflammatory reaction which was most commonly observed in the lung of the injected mice. In 8 instances all of the 5 mice which were used with each transfer showed evidence of pulmonary involvement at autopsy Six of the transfers failed to infect all of the mice, one or more being normal throughout at autopsy In one case, only a single mouse showed evidence of disease

Thirteen of the 14 mouse transfers were made from the Princeton strain of albino rat and one from the Cornell strain All of the rats used in these experiments were at least a year old

and all showed lung lesions at autopsy In one transfer from the Princeton strain the suspension was made from middle ear exudate

Transmission of the Experimental Disease in Mice by Nasal Instillation

The mouse disease experimentally induced through the agency of the rat was readily transmissible from infected to normal mice by the nasal instillation of lung suspensions Two separate injection series in mice originally begun with lung suspensions from the Princeton strain of rat were maintained for 8 and 13 passages, respectively The survivors of the 5 mice used with each passage were killed at intervals of at least 4 weeks after injection and invariably showed unmistakable evidence of the disease at autopsy The mouse disease originating from the Cornell strain of albino rat was also transmissible in series and was maintained for 5 passages

The Princeton strain of white mouse used in these and the subsequent experiments had been under close scrutiny for many years with no indication of respiratory disease Since the transmission experiments involved the serial passage of lung suspensions, a method employed for the detection of infective agents which are supposedly latent or inactive, parallel passage series were conducted with lung suspensions from normal mice Five mice removed at random from the stock colony were killed and a 10 per cent suspension prepared in saline from the whole, normal appearing, pooled lungs This was injected intranasally in 5 mice which were killed after 4 weeks and the same procedure continued. Two separate series embracing the entire period of the work on endemic pneumonia were carried out and maintained for 25 and 10 passages respectively The lungs of the 175 mice used in these tests were all normal at autopsy, at the end of the 4th week. In several passages a duplicate group of mice were injected and killed 7 to 10 days later The lungs of these mice were also normal

Transmission of the Experimental Disease in Mice by Direct Contact

Preliminary attempts to transmit the mouse disease by placing 5 normal mice in the same cage with a single infected host for 4 weeks were largely unsuccessful Thirteen of the 15 mice killed after a month's exposure were normal throughout at autopsy while 2 showed only an otitis Subsequent experiments indicated that the incidence of disease for this time interval was not significantly affected by the number of infected hosts to which the normal mice were exposed Increasing the proportion to equal numbers of each failed to increase the number of infected mice after 4 weeks of exposure The time elapsing between nasal injection of the infected hosts and their exposure to normal mice likewise failed to affect the rate of transmission It was equally low after a month in contact whether exposure was begun immediately after nasal injection of the infected mice, 2 weeks later, or 7 weeks later

The rate of contact infection was influenced, however, either by the duration of the exposure period or by the length of time the normal mice were held after exposure In 6 of 10 mice exposed for 8 weeks to an equal number of infected mice the middle ears were involved though the lungs were normal It is of

some significance that the normal appearing lungs from the mice with otitis were infective on subsequent nasal injection, while those from the otitis-free mice exposed for a period of 4 weeks were non infective The 4 survivors of 10 mice exposed for 12 weeks and then held for an additional 20 weeks showed extensive pneumonia at autopsy in addition to otitis.

Transmission from Sources Other Than the Lung and by Different Routes

It was previously noted that the experimental disease was transmissible to mice by the nasal injection of middle ear exudate from infected rats Middle ear exudate was similarly effective in its passage from mouse to mouse, as was also nasal exudate. In both cases the disease pattern was identical with that produced by lung suspensions Brain, spleen, and defibrinated blood from the heart of infected mice were innocuous, however, on nasal instillation

Attempts to reproduce the disease by routes other than the upper respiratory tract were uniformly unsuccessful Mice injected in the foot pad, heart, peritoneal cavity, or brain with active lung suspensions showed no specific response The intracerebral series included a second passage made with both Princeton and Swiss strains of mice The passaged brain was finally injected intranasally in Princeton mice with negative results

The Appearance of Lesions in Mice Killed at Varying Intervals after Nasal Instillation

No reaction was observed in the lung, middle ear, or nasal passages of mice killed on the 3rd and 7th day after injection The experimental disease was transmissible, however, from the normal appearing lungs removed on the 7th day

Pneumonia was first observed on the 9th day after injection, occurring in each of 5 mice, but otitis was delayed until the 21st day, when 2 of 5 mice showed exudate in the middle ears. Rhinitis was apparent in only one mouse of this series and was also observed on the 21st day Chattering was noted as early as the 7th day following nasal instillation but was not characteristic until the 9th to the 14th day

The Nature of the Experimental Disease in Mice

The following summary of the chief characteristics of the experimental disease is compiled from the records of several hundred infected mice examined at intervals over a period of 3 years but embracing all seasons of the year

Chattering.—The initial symptom was an audible intermittent chattering observed as early as the 7th day after the nasal instillation of 10 per cent suspensions but more commonly delayed until the 10th to the 14th day It became more prominent as the disease progressed and continued throughout the life of the host.

Pneumonia.—The rate of pneumonia in mice killed 4 weeks after injection was high—96

per cent in 100 mice. Macroscopically it was characterized by gray or pinkish gray areas of consolidation which commonly appeared first at the attachment of a lobe but also occurred marginally or at the tip The initial reaction remained localized or subsequently extended to include an entire lobe, the azygos and right middle being most commonly involved. The lobe at this time was generally contracted and under low magnification appeared uniformly consolidated or showed lobular areas of different consistency and color Abscess formation, cavitation, or the presence of a frank exudate on the cut surface of consolidated areas was not observed.

Microscopically the early reaction in which the lung showed isolated patches of consolidation was characterized by the presence of exudate composed chiefly of polynuclear leucocytes and often sparse in amount within one or more bronchioles The lymphocytic cells around the involved bronchioles were moderately increased in number Radiating outward from them were islands or strands in which the alveoli were completely filled with cells A large mononuclear epithelial like cell often predominated but polynuclear leucocytes were also commonly observed together with a few lymphocytes The adjacent lung tissue was normal in appearance

The later reaction with lobar consolidation showed a complete alteration in the microscopic structure of the lung The chief feature was an intense lymphocytic infiltration around all bronchioles which were often filled with a leucocytic exudate The adjacent lung tissue contained no open air spaces All of the alveoli were compressed and filled with cells, among which the large mononuclear cell was again prominent. No inclusion bodies were observed

Otitis Media.—The rate of middle ear involvement was also high—94 per cent in 100 mice At autopsy a turbid exudate variable in volume and consisting chiefly of polynuclear leucocytes was present in the tympanic cavity Generally there was no other indication of aural irritation, equilibrium was unaffected, but in a few instances the tympanic membrane was under pressure and bulged outward

Rhinitis.—The rate of nasal involvement was low—31 per cent in 100 mice Irritation of the upper air passages was judged by the presence or absence of a frank exudate removable with a capillary pipette When present the exudate varied in consistency but was generally a rather thick, white, granular material showing a predominance of polynuclear leucocytes An outward discharge from the nasal passages was never observed

General Reaction.—Prior to the onset of chattering and for some time thereafter infected mice commonly showed no other symptoms, though when weighed after 4 weeks, prior to autopsy, it was apparent that they had failed to gain weight normally As the disease progressed signs of constitutional disorder began to appear The infected mice were unthrifty in appearance, often gaunt, and showed ruffed hair A few mice held as long as a year continued to show the characteristic features of the disease. Transmission to normal mice was accomplished by nasal passage from the lung

Incubation Period.—The interval elapsing between nasal injection and the appearance of symptoms or pathologic changes varied from 7 to 14 days

Mortality.—The disease was accompanied by a specific mortality which increased as the disease progressed The overall rate for 65 mice maintained for 4 to 22 weeks after nasal instillation was 33 per cent The incidence of deaths in mice during the 4 to 5 week period was 5 per cent, whereas the rate during the 20 to 22 week period was 65 per cent.

DISCUSSION

The preceding experimental findings clearly indicate the regular establishment of a specific disease entity in mice by nasal transfer from the lung and the rarely involved middle ear of albino rats with endemic pneumonia The disease

thus induced in mice is characterized by a pneumonia which resembles that of the rat in its initial involvement of the bronchioles but differs in not progressing to bronchiectasis. Unlike the rat, the mouse also shows a high rate of middle ear involvement. The nasal response in the mouse is similar to that in the rat, being characterized by a variable but generally low rate of rhinitis. Symptomatically and pathologically, aside from its lower rate of nasal reaction, the mouse disease closely resembles infectious catarrh to which both the rat and the mouse are naturally subject. As will be shown later, however, the two diseases can be readily differentiated in the mouse by the microscopic examination of exudates

The induced disease is regularly transmissible from mouse to mouse by the nasal instillation of lung or exudate suspensions There is no indication that the development of the disease in mice is influenced by a latent infective agent present in the supposedly normal mice and activated by passage from lung to lung, the corresponding loci in a parallel passage series maintained in normal mice being unaffected.

Transmission by direct contact is also demonstrable but is less readily accomplished and requires a long exposure or maintenance period to bring out the full pattern of the disease The retarded development is probably correlated with the low rate of rhinitis and indicative of a meager output of the causal agent by way of the nasal passages. The only manifestation of the disease in mice exposed for 4 to 8 weeks is an otitis, the middle ears containing an exudate which is fully infective on subsequent nasal passage. The lungs of mice exposed for 8 weeks, though macroscopically normal, may likewise be infective. There would appear to be a difference in organ susceptibility between the lung and the middle ear, possibly related to an altered resistance due to the repeated inhalation of subinfective increments of the causal agent and capable of expression only in the lung In our experience no counterpart of the induced disease has been encountered in mice under natural conditions. For the disease to exist naturally it must be spread by direct contact. The long exposure period required for its development through cohabitation may be a limiting factor in this connection.

The disease established initially in mice by transfer from the rat and subsequently maintained by nasal transmission or direct contact is similar to infectious catarrh in its chronicity and slow inevitable progress from which there is no apparent recovery It is essentially pneumotropic, activity and growth of the causal agent being limited to the respiratory tract including the associated middle ears The distribution of lesions in the lung is, however, unlike that in infectious catarrh which may equally involve any of the five lobes In the present disease, while initial lesions may be present in any lobe, subsequent extension with complete lobar consolidation is generally limited to the right middle and azygos lobes. The evident inability of the causal agent to extend

from the primary focus in the other three lobes even after months of residence there is likewise suggestive of the interplay of an altered resistance

SUMMARY

A specific disease entity was established in white mice by the nasal instillation of lung and exudate suspensions from each of 14 adult albino rats affected with endemic pneumonia

The induced disease was characterized by pneumonia (96 per cent), otitis media (94 per cent), and rhinitis (30 per cent) It progressed slowly after an incubation period of 7 to 14 days and was attended by a variable mortality which reached 33 per cent in 22 weeks

Maintenance of the disease was regularly accomplished by direct transmission from mouse to mouse but only by the nasal route and only from the three foci of involvement It was also transmissible by direct contact but required a prolonged period of exposure

The relation of the disease to infectious catarrh, which it closely resembles, is discussed

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STUDIES ON ENDEMIC PNEUMONIA OF THE ALBINO RAT

II. The Nature of the Causal Agent in Experimentally Infected Mice

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Endemic pneumonia of the albino rat is commonly regarded as an infectious disease but its etiology is still uncertain, though it has often been investigated and a wide variety of bacteria isolated from the lung The opinion has also been expressed that the disease is not primarily bacterial in origin but is referable rather to a non-specific pulmonary irritation with resultant bronchial obstruction

Associated Bacterial Factors —Klein (1) isolated a diphtheroid which he named *B muris* from the lung and heart's blood of two albino rats with pneumonia accompanied by hemorrhage and visceral hyperemia. Subcutaneous injection of the bacillus in rats resulted only in a local abscess Mitchell (2) studied a highly fatal outbreak of pneumonia in albino rats and isolated the same organism from the lung It likewise produced a local abscess on subcutaneous injection and resulted in death with pleural involvement on intrapleural injection It was innocuous, however, when introduced by the nasal route.

Pneumonic lesions similar to those in endemic pneumonia of the albino rat have also been reported in wild rats. The morbidity rate, however, is much lower in the wild rat. Tunnicliff (3) reported one case of pneumonia in 28 wild rats, and we (4) observed six cases in 58 rats Wolff (5) isolated a hemophilic influenza-like bacillus from wild rats with lesions in the lung On injection in the rat by various routes including intratracheal, it was toxic and often resulted in death but was not recoverable from the organs at autopsy

Tunnicliff (3, 6) observed a Gram negative streptothrix in 56 films from the lungs of 60 white rats with bronchopneumonia and isolated it in culture from 20 Cultures were injected in young rats but only intraperitoneally and with irregular results. One rat died 24 hours later with acute lesions in the lung from which the streptothrix was reisolated Hoskins and Stout (7) studied a distemper-like rat disease which was often accompanied by pneumonia In about half of the cases they isolated *B bronchisepticus* from the lung heart's blood, and nasal passages The pathogenicity of the organism was not studied

Jones (8) recovered a pleomorphic organism with terminal clublike, capsular swellings from the lungs of white rats with pneumonia It closely resembled *Actinobacillus actinoides* previously described by T Smith (9) as the probable cause of a pneumonia in calves. Because of the high natural rate of pneumonia in the available rats, it was impossible to evaluate the relation of the organism to the disease Meyer

and Batchelder (10) in their studies on plague observed an accidentally initiated epidemic in albino rats of hemorrhagic septicemia with pulmonary involvement The associated and highly pathogenic organism, which also occurred in wild rats, was named *Pasteurella muricida*

Nelson (11) studied the bacterial flora of the lungs from 40 albino rats with pneumonia *Actinobacillus actinoides* (*muris*) was most commonly encountered (35 per cent) as it also was from middle ear exudates (43 per cent) in otitis which at that time was believed to be a frequent accompaniment of the pneumonia. A pathogenic diphtheroid resembling *B muris* of Klein was also isolated from the lung and middle ear but only occasionally It is of interest that 42 per cent of the lung cultures showed no growth on blood-enriched agar plates Smith, Bethune, and Wilson (12) isolated an unidentified Gram-positive anaerobic coccus from the lungs of 16 white rats with pneumonia and a motile, Gram-negative aerobic bacillus from 10 Pure cultures of these bacteria were innocuous on intratracheal injection Eleven of 17 young rats with no roentgenographic indication of pneumonia injected *via* the trachea with a mixture of the two bacteria showed evidence of pulmonary involvement at autopsy 9 to 21 days later

Klieneberger and Steabben (13) noted that rats with pneumonia were more often carriers of the pleomorphic organism *Streptobacillus moniliformis* than were normal rats They also noted the similarity between *Streptobacillus moniliformis* and the organisms described by Tunnicliff, Jones, and Nelson, and suggested their identity They isolated a pleuropneumonia-like organism, which resembled the earlier one described by Klieneberger (14) as a symbiont of the streptobacillus, from the lungs of 17 out of 19 rats with pneumonia In a later and more extensive study Klieneberger and Steabben (15) recovered the same pleuropneumonia-like organism, now termed L3, from the lungs of 138 out of 251 albino rats It was found in the lung of young rats in the absence of macroscopic lesions and the rate of its isolation increased as the rat matured Because of the high rate of carriage no definite evidence could be obtained of its etiological relation The suggestion was made that an adjuvant factor might be required to establish the organism as a pathogenic agent Warren (16) in the study of another species of the pleuropneumonia-like organism, the Woglom-Warren strain of L4, observed that nasal instillation in adult rats resulted in an acute interstitial pneumonia with mononuclear leucocytes as a prominent factor

Associated Non-Bacterial Factors—Passey, Leese, and Knox (17) suggested that since lack of reasonable exercise deprived the rat of full use of the lung, the resulting stagnation of normal mucous secretion might result in dilatation and mild sacculation with subsequent bacterial contamination The continued inhalation of dust particles from litter and feed has also been noted in unpublished comments as a possible initiating factor

The Bacteriological Examination of Selected Albino Rats with Pneumonia

Our early work with endemic pneumonia was carried out with rats which when mature showed a high rate of otitis in addition to pneumonia The frequent isolation of *Actinobacillus muris* from both loci, particularly the middle ear, coupled with the observation that it produced an inflammation

within the tympanic cavity on intraaural injection led us (18) to believe that it was of etiological significance. The subsequent examination of rats from the so called selected colony originally established with disease free breeders clearly indicated, however, that *Actinobacillus muris* was merely a secondary invader and that the high rate of otitis observed prior to selection was not associated with the pneumonia (19) Middle ear involvement was reduced to a low level in the selected rats and to date has never exceeded 4 per cent whereas the pneumonia was not significantly affected and is still high in mature rats With the reduction in otitis, *Actinobacillus muris* disappeared from the colony and has not been reintroduced. All attempts to cultivate it from the lung and occasional middle ear exudate have been unsuccessful though from time to time it has been isolated from rats obtained elsewhere.

As previously noted, a high rate of otitis is characteristic of infectious catarrh which, it may be presumed, was present in the original rat colony together with endemic pneumonia but unlike the latter was eliminated by selective breeding The coccobacilliform bodies which are the cause of infectious catarrh have never been demonstrated in exudates or tissues from the selected rats.

Many attempts were also made to recover pleuropneumonia like organisms from the lungs of selected rats with pneumonia using media known to support growth of these organisms but were uniformly unsuccessful.

Brucella bronchiseptica was occasionally isolated from the lung of pneumonic selected rats, but neither it nor any other cultivable organism was recovered sufficiently often or in sufficient numbers to be of etiological importance

The Bacteriological Examination of Experimentally Infected White Mice

The transmission of a communicable respiratory disease to mice (20) by the nasal instillation of lung suspensions from rats with pneumonia clearly indicated the association of an infective agent and provided an additional means of isolation and study The resemblance between this disease in the mouse and infectious catarrh to which it is naturally subject has already been noted The most dependable guide in the diagnosis of infectious catarrh is the microscopic examination of exudate from the middle ears and nasal passages, Gram-stained films regularly showing extra or intracellular coccobacilliform bodies. Tissue and exudate films from mice with the experimentally induced disease were repeatedly examined but morphologic units resembling the coccobacilliform bodies were never observed This difference between the two diseases when examined in parallel series was particularly striking

Microscopic and cultural examination of tissues and exudates from mice with the induced disease was also carried out with the view of demonstrating pleuro pneumonia like organisms

The medium most commonly employed in the examination of both mice and rats was nutrient agar, pH 7.6 to 8.0 enriched with serum from defibrinated horse blood the concentra

tion being approximately 30 per cent. Ascitic fluid and horse serum fortified with an equal volume of a 10 day egg embryo extract were also used in some instances Petri dishes of these media were inoculated fairly heavily and then sealed with scotch tape The inoculum included 10 per cent suspensions of lung in saline solution and exudates both undiluted and on dilution with saline The plates were incubated at 37°C for 7 to 14 days and inspected at intervals under low power magnification Films of exudate were examined microscopically after staining with Gram and Giemsa, respectively

Colonies of pleuropneumonia-like organisms were never demonstrated on inoculated horse serum agar or ascitic fluid agar plates at any stage of the disease During part of the examination a member of this group of organisms isolated from another source was maintained for many transfers on similar media The microscopic examination of exudates likewise failed to show any of the morphologic forms characteristic of the pleuropneumonia-like group The inoculated plates generally showed few bacteria of any species and none were isolated which were of any etiologic significance

Titration of the Infective Agent in Lung Suspensions from Mice

Titrations were made to determine the minimum infective dose of the unknown agent present in the lung of mice with the induced disease. The results of four experiments were identical in establishing 10^7 as the highest dilution of the lung which retained activity on subsequent nasal instillation in mice Each of the four original suspensions produced lung lesions in all of the injected mice through dilution 10^5 In the higher dilutions there were individual differences which are summarized in Table I It is of some interest that in the upper dilution range a number of the mice showed involvement only of the middle ears

In the above experiments the pooled lungs, comprising both normal and involved tissue, from mice killed about 4 weeks after nasal instillation were weighed and ground in a Waring blendor with sufficient buffered saline (pH 7 4) to make a final concentration of 10 per cent. The resulting suspensions were centrifuged at low speed for 15 minutes to remove coarser particles and subsequently diluted at tenfold intervals from 10^1 through 10^8 The volume of inoculum was included in the final dilution Unless otherwise noted, the same procedure was followed in all of the subsequent titrations Five mice weighing 15 to 18 gm were injected intranasally while under ether anesthesia with 0 05 ml of the respective dilutions and autopsied 4 weeks later The condition of the lung under low power magnification was used as an index of infection If the lung was normal the middle ears were exposed and examined for the presence of exudate

Filtrability of the Infective Agent in Lung Suspensions from Mice

Qualitative filtration experiments with lung suspensions indicated that Berkefeld V filters were generally permeable to the unknown agent but that N filters were not The V filtrates, in a dilution of 2×10^2, of 3 out of 4 lung suspensions were infective for mice on nasal instillation while equivalent dilutions of the 4th V filtrate and of 3 N filtrates were innocuous There was

a considerable quantitative loss in the activity of the lung suspensions after passage through V candles In two experiments the titer of the infective agent was reduced from 10^7 to 10^4 and 10^5, respectively The limiting dilutions of both filtrates produced lung lesions in only one of the 5 mice which were injected *None of these mice showed involvement of the middle ear, but an* otitis in the absence of pneumonia was observed in other mice of the same series.

The suspensions used in the filtration tests were made from the pooled and entire lungs of mice killed 4 weeks after nasal instillation The lung tissue was finely ground either in a glass tissue grinder or in the Waring blendor and sufficient nutrient bouillon added to make a final concentration of 10 per cent. The suspensions were centrifuged at low speed for 15

TABLE I

The Response of Mice to Diluted Lung Suspensions Injected Intranasally

Suspension No	No of mice with each dilution	Dilution							
		10^5		10^6		10^7		10^8	
		Pneumonia	Otitis	Pneumonia	Otitis	Pneumonia	Otitis	Pneumonia	Otitis
1	5	5*		5		5		0	0
2	5	5		4	1	1	0	0	0
3	5	5		5		0	1	0	0
4	5	5		1	2	2	0	0	0

* If the lung showed involvement the middle ears were not examined.

minutes and the supernatants filtered through number 3 V and N Berkefeld candles. The filtrates showed no colony growth on sealed 30 per cent horse serum agar plates.

Sedimentation of the Infective Agent by Centrifugation

The inability of the infective agent to pass through N Berkefeld filters suggested that its particle size might be comparable to that of the pox viruses. Accordingly, experiments were carried out to determine whether it simulated the elementary bodies in its response to centrifugation Infective lung suspensions were spun at approximately 3500 R.P M. in a small angle centrifuge and at 3700, 5000, and 9000 R.P.M. in a Pickels type centrifuge. Qualitative tests of the respective sediments and supernatants in mice indicated that the infective agent was present in both fractions at speeds through 5000 R.P.M but only in the sediment at 9000 R.P.M

Quantitative tests were then made with 3 different lung suspensions, the initial concentration of the infective agent being compared with that of the supernatants and sediments after centrifuging at 9000 R P.M In 2 of the suspensions which titered 10^6 and 10^7, respectively, before centrifugation the

infective agent was present in equivalent concentration in the sediments but was not demonstrable in the supernatants. In the third suspension, which likewise titered 10^7 initially, all of the agent was not removed by centrifuging, the titers of the sediment and supernatant being 10^6 and 2×10^2 respectively. In this experiment, which is summarized in Table II, some of the mice which received higher dilutions of the inoculum again showed middle ear involvement in the absence of pneumonia.

Pleuropneumonia-like colonies were not demonstrable on serum agar plates inoculated with sediments prior to resuspension, nor were the characteristic morphologic units observed in Giemsa- or Gram-stained films. Sediment films impregnated with silver by the Morosow method did show, however, intensely black and sharply outlined spherical particles which were smaller than the elementary bodies of vaccinia. This oberservation was of some interest since the sedimentation tests indicated that the infective agent, while a

TABLE II

Titration of the Infective Agent before and after High Speed Centrifugation

Nature of the suspension	No. of mice with each dilution	No. of mice infected with each dilution						
		2×10^2	10^3	10^4	10^5	10^6	10^7	10^8
Before centrifugation	5			5	5	3*	2	0
Sediment at 9000 R.P.M.	5			5	5*	2	0	0
Supernatant at 9000 R.P.M.	2	2	0	0	0			

* 2 mice showed otitis only.

relatively large sized particle, was probably smaller than the elementary bodies. The sediments were not sufficiently pure, however, to warrant identification of these particles.

In the preceding centrifugation tests 10 per cent suspensions of pooled entire mouse lungs were prepared in buffered saline by grinding for several minutes in a Waring blendor, the total volume being 50 ml. After centrifuging at low speed for 15 minutes, 15 ml. amounts of the supernatants were spun at high speed for 30 minutes. On removal of the supernatant the small amount of sediment was resuspended in sufficient buffered saline to restore the original volume. The original suspension and the two fractions after high speed centrifugation were tested in mice by nasal instillation. Before the sediments were resuspended they were inoculated on sealed 30 per cent horse serum agar plates which were incubated for 7 to 10 days. Dried films were also stained by the Giemsa, Gram, and Morosow methods.

Cultivability of the Infective Agent in Embryonated Eggs

Attempts to cultivate the infective agent in embryonated eggs by chorio-allantoic, amniotic, and yolk sac inoculation were uniformly unsuccessful. Aside from a few eggs that were contaminated with bacteria, the embryos were normal and active through the 7th day of incubation after inoculation and

no pathologic changes were apparent in the supporting membranes. Tissue suspensions made after several blind passages were inactive on nasal instillation in mice.

Bacteria free lung and exudate suspensions, active sediments, and active V filtrates were used in the inoculation of eggs. 0.05 to 0 1 ml. of the inoculum was injected in 10 day embryonated hen s eggs by way of the chorioallantoic membrane and amniotic cavity and in 6 day eggs by way of the yolk sac. The inoculated eggs were incubated at 37°C and opened on the 3rd to 7th day After a number of passages, which varied from 2 to 10 suspensions were made from the chorioallantoic membrane and yolk sac and injected intranasally in mice

Viability of the Infective Agent on Storage at Low Temperatures

The infective agent was unstable under ordinary conditions of refrigeration, 10 per cent lung suspensions in buffered saline being inactivated after a week at 40°C In a frozen state under dry ice it remained viable, however, for a period of at least 13 weeks

Identity of the Agent in Experimentally Infected Mice

Although no certain test for identifying the infective agent was devised and the supporting evidence was largely circumstantial, it seems permissible to conclude that the same agent was present in all experimentally infected mice and was originally transmitted to them from the rat. The behavior of the agent in respect to its localization in the host was sufficiently characteristic to serve as a partial guide Supplementary information was supplied by the microscopic examination of exudates and the cultural examination of lung suspensions. Instances in which injected mice showed involvement only of the middle ear were followed by an additional passage which always brought out the full pneumotropic activity of the agent. As indicated in the preceding paper (20) the serial passage in mice was adequately controlled by the parallel examination of normal mice which yielded no counterpart of the infective agent.

DISCUSSION

It is difficult to appraise much of the early work on endemic pneumonia of the albino rat because the criteria for judging the significance of a given agent were inadequate. It is our opinion that any unknown agent should meet certain requirements in addition to the original Koch postulates to establish a causal relation to the disease in question It is essential that the agent reproduce the basic features of endemic pneumonia when introduced in a pure state by way of a natural portal of entry, which in all probability is the upper respiratory tract Moreover, the agent should be experimentally transmissible by nasal passage and naturally so by cohabitation None of the described bacteria have met these requirements and it seems justifiable to conclude that the etiology of endemic pneumonia is still uncertain. Some of the associated microorganisms,

as *Brucella bronchiseptica* and *Pasteurella muricida*, undoubtedly produce a pulmonary reaction in the rat but are unrelated to endemic pneumonia and if isolated from the lung of infected rats are present only as secondary invaders

Critical study of endemic pneumonia has been particularly handicapped by the lack of suitable rats, the disease being universally distributed in so far as is known An additional source of error has been the coexistence of infectious catarrh which may simulate endemic pneumonia in its initial involvement of the lung By using specially selected rats we were able to eliminate infectious catarrh and also to rule out *Actinobacillus muris* (*Streptobacillus moniliformis*) as a significant factor but still had to contend with a high rate of endemic pneumonia in adult rats The disease may also appear in younger animals but in general the immature rat fails to show macroscopic evidence of pneumonia under natural or experimental conditions of infection and appears to be refractory It was for these reasons that an alternative host was sought and the mouse finally chosen

It was subsequently found that an infective agent was regularly present in the lungs of adult selected rats with pneumonia, which on transfer to mice likewise gave rise to a pulmonary inflammation Bacteriological examination of lung suspensions and exudates from experimentally infected mice as well as from naturally infected selected rats failed to demonstrate any of the bacteria isolated by previous workers Because of the high incidence of pleuropneumonia-like organisms reported by Klieneberger and Steabben (15) in the pneumonic lungs of rats, particular attention was paid to these bacteria It has already been noted that the morphologically similar coccobacilliform bodies of infectious catarrh were not demonstrable in the experimentally infected mice The repeated attempts which were made to cultivate pleuropneumonia-like organisms using adequately enriched media and to demonstrate them in films were likewise uniformly unsuccessful

In view of the inability to identify a cultivable organism with the infective agent, experiments were carried out to determine whether it had the characteristics of a virus The results indicated that the agent was filtrable, though not readily so, and suggested that it was a particle somewhat smaller than the elementary bodies of vaccinia. Like the elementary bodies it was refractory to staining with the usual aniline dyes, but unlike them was not cultivable in embryonated eggs nor able to withstand a temperature of 40°C for a period longer than a week These findings while broadly suggestive of a virus might likewise be applicable to a pleuropneumonia-like organism The only differential characteristics at present are the inability of the agent to multiply in a cell-free medium and its tinctorial properties Since these characteristics might be referable to technical inequalities, it seems advisable to regard the agent as virus-like in nature and defer a final decision until additional observations have been made of its behavior

The virus-like agent fulfills the essential postulates for identifying it as the specific cause of the experimentally produced mouse disease These postulates have not been fulfilled for similarly identifying it with the rat disease and until this is done it can be concluded only that the agent is a frequent resident of the lung in endemic pneumonia.

SUMMARY

Coccobacilliform bodies, pleuropneumonia like organisms or other cultivable pathogenic bacteria were not demonstrable in lung and exudate suspensions from selected adult rats naturally infected with endemic pneumonia or from experimentally infected mice.

The specific factor in lung suspensions from mice was identified with an agent which was active on dilution through 10^7 It failed to pass through Berkefeld N filters but was generally present in V filtrates, with a loss in titer up to 1000-fold It was largely removed from suspension on centrifugation at a speed of 9000 R.P.M for 30 minutes.

Attempts to cultivate the agent in embryonated eggs were uniformly unsuccessful In fluid suspension it failed to withstand storage at 40°C for a week but remained viable for at least 3 months in a frozen state under dry ice

The relation of the infective agent to viruses and to pleuropneumonia like organisms is discussed

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INFLUENCE OF EXPERIMENTAL KIDNEY DAMAGE ON HISTOCHEMICALLY DEMONSTRABLE LIPASE ACTIVITY IN THE RAT COMPARISON WITH ALKALINE PHOSPHATASE ACTIVITY

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PLATES 1 and 2

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The introduction of methods capable of demonstrating both alkaline (1, 2) and acid phosphatase (3) in tissue sections has led to interesting results concerning the distribution of these enzymes The kidney was found to contain a large amount of alkaline phosphatase, predominantly in the cortical portion (2, 4–6) Under various experimental conditions changes in the amount and distribution of alkaline (7–12) as well as of acid phosphatase (11) have been described

In the present investigation, lipase activity was studied in the normal kidney of different animals and under varying experimental conditions in the rat, using Gomori's recently published technique (13) Sections were also stained for alkaline phosphatase activity in order to compare the influence of experimental damage on two different, microtechnically demonstrable enzymes.

Material and Methods

Kidney sections from normal dogs and rats of the Wistar strain rabbits, mice, hamsters and guinea pigs were used Kidney damage in rats was caused by subcutaneous injections of mercury bichloride and uranium nitrate, dietary choline deficiency and ligation of the ureter

Lipase activity was demonstrated by Gomori's method with some modifications. This technique is based on the deposition of insoluble calcium palmitate or stearate if the sections are incubated with a water-soluble ester of palmitic or stearic acid in the presence of calcium chloride. The calcium palmitate formed at the site of lipase activity is visualized by transforming it into its lead salt and demonstrating the lead by changing it into dark brown sulfide. The tissue was fixed in ice cold acetone for 24 hours, with one or two changes of the acetone, placed for 24 hours in absolute alcohol, and left for 1 to 2 hours in xylol and for another 2 hours in paraffin at 56 C. By using this procedure, serial sections can be stained for lipase as well as alkaline and acid phosphatase activity Instead of a maleate buffer the acetate barbital sodium buffer of Michaelis (14) was used in the incubation mixture. 5 parts of a solution containing 9 714 gm. sodium acetate, $3H_2O$ and 14.714 gm barbital-sodium (sodium diethylbarbiturate) in 500 cc. CO_2-free water 5 parts of N/10 hydrochloric acid, and 10 parts distilled water will give a suitable buffer solution of pH ±7 4 Tween 40 (sorbitan monopalmitate, Atlas Powder Co., Wilmington Delaware) served as substrate. The slides were

incubated from 40 to 48 hours at 37°C Each slide also contained liver tissue The latter shows a constant amount of lipase activity (13) and served as a good control for the quality of the preparation

Phosphatase activity was demonstrated with Gomori's technique as modified by Kabat and Furth (5) with minor alterations (11) No counter stain was used In order to visualize preformed calcium, which in most cases cannot be distinguished in sections prepared for the demonstration of phosphatase activity, the following technique was used Slides were covered in the usual way with a 0 25 per cent solution of celloidin in equal amounts of absolute alcohol and ether and placed for 15 minutes in a 2 per cent solution of cobalt nitrate. After washing in tap water, they were immersed for 1 minute in a dilute solution of ammonium sulfide This stained most of the preformed calcium deposits black After washing in tap water, the slides were brought into the incubation mixture used for demonstration of alkaline phosphatase activity A few drops of dilute ammonium sulfide were added to this mixture The slides were then incubated for 10 to 14 hours at 37°C They were, after washing, placed for 15 minutes in a 2 per cent cobalt nitrate solution The formed cobalt phosphate could be demonstrated by covering the slides with a saturated alcoholic solution of dithiooxamide (rubeanic acid—Eastman Kodak Co) to which a few drops of ammonia water were added Cobalt forms complex salts of rubeanic acid, which are insoluble in water and dilute mineral acids, and appear as intensely colored yellow-brownish precipitates (15) The preformed calcium which has been stained black contrasts well with the yellowish brown cobalt complex salt of rubeanic acid signifying phosphatase activity Gomori's method (16) for simultaneous staining of preformed calcium and phosphatase activity could not be used since acridine red Gruebler, essential for this technique, could not be obtained

Lipase Activity in the Kidneys of Normal Animals

All the species examined showed evidence of lipase activity It was moderate in the kidneys of guinea pigs, rabbits, hamsters, and mice, irregularly distributed in the kidneys of dogs, and present in large amounts in the kidneys of rats It was found only in the cortical portion of the kidney and was restricted to the proximal convoluted tubules (Fig 1) It was present only in the cytoplasm and not in the nuclei In contrast to the appearance of alkaline phosphatase, it was not concentrated at the brush border but diffusely distributed throughout the cytoplasm of the cells (Fig 2) The deposits of lead sulfide, signifying lipase activity, were granular but not infrequently formed fine needles The deposits, in general, seemed to be more distinctly granular when barbital was used instead of the maleate buffer Occasionally, the epithelium lining of the kidney pelvis showed some activity The distribution of lipase activity in the cortex of the proximal convoluted tubules corresponded closely to that found for alkaline phosphatase The latter is also found predominantly in the proximal convoluted tubules Alkaline phosphatase activity, however, is found not only in the cytoplasm but also, to some extent, in the nuclei of the convoluted tubules Phosphatase activity also appears in the cytoplasm of the ascending limbs of Henle in the dog (4), mouse, and rabbit (9) In the rat a very extensive reaction is seen in most of the tubules situated in the cortex Menten, Junge, and Green (17) obtained a similar picture by

using a histochemical azo dye test. They concluded that in the rat kidney the cytoplasmic phosphatase is found not only in the proximal convoluted tubules but also in the loops of Henle and in the distal convoluted tubules. However, in our preparations of normal rat kidneys, even after long incubation no cytoplasmic activity was found in the tubules situated in the medulla where the loops of Henle can easily be identified There was, however, a varying amount of phosphatase activity in the nuclei of most of the tubules as well as of the capillaries in the medulla The glomeruli frequently exhibited pronounced activity in the rat (11), mouse (17), and dog

Effect of Poisoning with Mercury Bichloride

Eleven rats varying from 150 to 250 gm. were given one subcutaneous injection of 1 to 2 mg mercury bichloride in a 4 per cent solution Five animals were sacrificed or died after 3 to 5 days These animals showed acute changes while those sacrificed later showed changes which obviously represented a subacute or healing stage. In the acute phase, extensive necrosis of the convoluted tubules was seen In many of the necrotic tubules, no cellular details could be recognized, while in others the degenerative changes were less severe Frequently necrotic cells separated from the basal membranes and were expelled into the tubular lumen In many of the excretory tubules, granular material was seen In two kidneys frequent mitotic figures were noticed The glomeruli were essentially normal. In four out of five kidneys calcification was seen which was very extensive in three cases

Suzuki (18) on the basis of vital staining with carmine, localized the damage in the last third of the convoluted tubules in the rabbit. Simmonds and Hepler (19), using the fat content of the terminal portion of the convoluted tubules as a means of identification confirmed these findings. Edwards (20) by teasing the macerated kidney of different animals under the microscope, found however that the damage was mainly localized in the second and third quarters of the convoluted tubules and that the most distal portion remained frequently undamaged

The kidneys of three animals sacrificed after 8 to 15 days showed only focal changes. Some of the cells in the convoluted tubules had an irregular appearance with prominent hyperchromatic nuclei A few necrotic tubules were still present In the deeper portion of the cortex near the medulla, occasional tubules were dilated and had a somewhat atrophic epithelium. In the kidneys of the three remaining animals, killed after 13 to 18 days, the changes were much more distinct. Focal dilation of the convoluted tubules was seen in many places mostly near to the medulla In these tubules, the epithelium was flattened and appeared atrophic. Occasionally, a few more normal appearing cells were interspersed in a row of markedly atrophic cells. In one case, interstitial areas of fibroblastic proliferation were observed Occasionally, tubules still contained frankly necrotic cells. Small deposits of calcium were

seen only in one case Few of the collecting tubules contained casts The glomeruli were essentially normal

Lipase Activity—In the kidneys of animals showing acute changes, lipase activity was only moderately decreased Desquamated necrotic cells still contained the enzyme, but took the stain diffusely There was, however, moderate to marked depletion in some of the damaged convoluted tubules Occasional lipase activity occurred in the tubular casts

In the subacute cases depletion of lipase activity was obvious in the atrophic and dilated tubules wherever changes of these sorts were found in hematoxylin-eosin preparations (Figs 3 and 4) Occasionally, the dilated tubules contained flat cells devoid of lipase activity intermingled with more normal appearing cells showing distinct activity

Phosphatase Activity—The changes in alkaline phosphatase activity were quite similar to those in lipase activity There was moderate depletion of the kidneys showing acute changes As previously described by Hepler and his coworkers (7, 21, 22), the necrotic tubules were frequently diffusely stained and desquamated cells were still active though often less intensely so than undamaged tubules The chromosomes of mitotic figures showed activity, as previously described for the dividing cells in other tissues (22–24) Amongst many casts, occasional ones were positive

By using the technique which permits a simultaneous demonstration of preformed calcium and phosphatase, it could be seen that necrotic, phosphatase-containing cells were the seat of beginning calcification As the calcification progressed more of the phosphatase disappeared In segments of tubules that were extensively calcified, stainable phosphatase could no longer be discerned

In the subacute cases, marked depletion was seen in the atrophic and dilated tubules while the tubules which appeared normal in preparations stained with hematoxylin and eosin showed a normal amount of enzymatic activity

Effect of Poisoning with Uranium Nitrate

The anatomical changes observed in the kidneys of various animals receiving uranium nitrate including the rat, have been described by Suzuki (18), Oliver (25), and Breedis, Flory, and Furth (8) in connection with the changes in alkaline phosphatase activity

Ten albino rats varying from 150 to 325 gm were used They were given a single dose of uranium nitrate varying between 1 5 and 3 mg subcutaneously Four animals that died or were killed 2 to 3 days after the injection showed extensive necrosis of the tubular epithelium The damage was located primarily in the distal portion of the proximal convoluted tubules, although it extended into Henle's loops and even into the distal convoluted tubules Mitotic figures were quite frequent in the epithelium of the proximal convoluted tubules Many of the distal convoluted tubules and excretory ducts contained granular material derived from the destroyed epithelial cells Deposition of

calcium mostly occurring in the distal portions of the proximal convoluted tubules was observed in one animal within 2 days after the injection

Six of the animals were killed after 7 to 25 days Their kidneys showed various degrees of damage Necrotic cells were still noticeable Some tubules showed mitotic figures and in addition, in some of the tubules, multinucleated cells were quite prominent. These cells, according to Oliver (25) are concerned with the regeneration of the necrotic epithelium Hyaline casts lying in the distal convoluted tubules and excretory ducts were found in several kidneys Many of the proximal convoluted tubules were markedly dilated The epithelium was flattened In other areas, the epithelium of the proximal convoluted tubules appeared atrophic, with a small or even collapsed lumen Inflammatory changes and proliferation of the connective tissue accompanied the atrophy of these tubules as pointed out by Suzuki and Oliver The glomeruli showed only little changes

Lipase Activity—In the acute cases, there was moderate depletion of lipase in the necrotic tubules In some the necrotic cells took the stains diffusely Occasional necrotic cells showed very little enzyme while the undamaged cells contained a normal amount.

In the kidneys of the six rats which were sacrificed after 7 to 25 days, the depletion was quite distinct wherever tubules were dilated and showed atrophic epithelium Frequently these epithelial cells were free from any activity The activity in normal appearing cells varied, mostly they showed a normal amount, but occasionally, it was depressed Many of the casts showed conspicuous lipase activity The intense staining reaction in these casts was not due to calcium deposits, since calcium stains in control sections were negative

Phosphatase Activity—The changes in phosphatase activity resembled closely those described by Breedis, Flory, and Furth (8) In the acute stage there was diffuse staining of necrotic tubules and moderate depletion in some of them Casts were frequently positive. In the more chronic cases there was marked depletion in the atrophic and regenerating epithelium Mitotic figures showed phosphatase activity Most of the glomeruli showed strong phosphatase activity As in mercury poisoning, calcium was deposited in necrotic cells which frequently still contained abundant phosphatase

Effect of Choline Deficiency

The morphological changes occurring in choline deficiency have been described previously (11 26-28) In the acute stage they consisted mainly of necrosis of the cortical tubules and degenerative changes in tubules not completely necrotic, with accumulation of extravasated blood between the connective tissue of the capsule and the outer surface of the cortex. In addition intense hyperemia of the capillaries of the cortical portion of the kidney with blood extravasation, was a very prominent feature in many areas. Acidophilic material in the form of casts was found in the lumen of Henle s loops, distal convoluted tubules, and most extensively in the collecting tubules.

The kidneys of seven animals with acute changes and of six with subacute, were available for study The diets used for the production of the typical kidney lesions have been reported previously (11) The highest incidence was observed in male weanling rats, given an experimental diet containing 8 to 10 per cent of fibrin and 0 3 per cent cystine as protein source

Animals which were sacrificed after 50 days on the experimental diet presented changes which were designated as subacute In this stage, the extravasated blood and the congestion had disappeared Many of the cortical tubules were atrophic and their cytoplasm was strongly reduced There was marked condensation and proliferation of the connective tissue stroma around the atrophic tubules and also fibrosis of the renal capsule Only a few casts had remained Occasionally the collecting tubules were dilated In some cases, only small foci of atrophic tubules were observed, while in others most of the cortical tubules were atrophic and only small clusters of normal appearing convoluted tubules remained

Lipase Activity —In the acute stage, lipase activity showed marked reduction which was most evident in the outer portion of the kidney cortex, where necrosis of the tubules was most prominent (Fig 5) Many of the necrotic tubules showed no activity while others revealed some Occasionally, necrotic cells showed diffuse brownish staining Reduction of lipase activity was found not only in completely necrotic tubules but also in tubules that were not so severely damaged as evidenced by their still intact nuclei The degree of reduction in lipase activity was not uniform and varied in different fields Most of the casts in collecting tubules were devoid of activity although an occasional one showed faint staining

In the subacute stage the lipase stain revealed marked depletion in the atrophic tubules (Fig 6) Most of them were completely devoid of lipase while occasionally some of them showed faint traces of the enzyme Convoluted tubules showing a normal appearance in hematoxylin and eosin stains also revealed a normal amount of lipase activity

Phosphatase Activity —The changes in phosphatase activity have been described previously (11) In the acute stage, marked reduction was found in the necrotic cortical tubules The degree of reduction varied in different cases and also in different sections of the same kidney Some of the casts filling the tubular system showed staining

In the subacute stage, the depletion of phosphatase in the atrophic tubules was even more distinct Only the convoluted tubules that appeared normal in hematoxylin and eosin preparations showed a normal amount of phosphatase activity Most of the glomeruli showed very marked activity

The Changes in Hydronephrosis

In twelve adult white rats, the left ureter was ligated The animals were killed 10 to 12 days after the operation Among them, five showed mild to

moderate and seven marked hydronephrosis In stained sections, there was dilation of the tubular system extending to the outer portion of the cortex in those kidneys in which marked changes were present. The capsular spaces of the glomeruli were frequently distended. The extent of the dilation was not uniform It was marked in some areas and less in others The epithelium in dilated tubules was flattened

Some kidneys showed collapse of some of the proximal convoluted tubules This phenomenon has been described in detail by Suzuki (18) who distinguished three periods in experimental hydronephrosis of the rat and rabbit. The first period, after ligation of the ureter, is characterized by the general dilatation of the tubular system and the capsular glomerular spaces. This is followed by a second period, characterized by the collapse of the proximal convoluted tubules and the loops of Henle In the third period, starting about 4 weeks after the ureteral ligation, collapse of the distal convoluted tubules and collecting tubules occurs

In kidney sections stained with hematoxylin and eosin, many of the cells in dilated as well as collapsed convoluted tubules have lost the eosinophilia of their cytoplasm In kidneys with only mild changes, the dilatation of the tubules was much less marked and much less extensive. In two kidneys there was subacute inflammation and marked fibroblastic proliferation beneath the epithelium of the kidney pelvis In all cases, the right kidney was entirely normal

Lipase Activity—The kidneys which showed marked hydronephrotic changes revealed considerable depletion of lipase activity (Figs 7 and 8) This depletion was seen in cells of dilated as well as of collapsed tubules. Occasionally, the epithelium in dilated tubules still showed some lipase activity Even in kidneys showing conspicuous hydronephrotic changes, the depletion was not evenly distributed and in some fields there were still a number of tubules showing normal amounts of lipase activity In the kidneys with only mild hydronephrotic changes the loss of enzymatic activity was much less pronounced. It was focal in distribution, mostly in tubules showing some dilatation The amount of lipase activity in the undamaged right kidney was normal in each case

Phosphatase Activity—Slides from each case were incubated for 2 and 12 to 14 hours. In full agreement with Wilmer's findings (9, 10) there was marked depletion in many dilated as well as collapsed proximal convoluted tubules In the kidneys with mild hydronephrosis, the depletion was much less marked and only focal The depletion of phosphatase activity was very pronounced in sections incubated for 2 hours This incubation time was also used by Wilmer In slides incubated for a longer time the depletion was still quite marked although in tubules in which the enzyme had disappeared from the cytoplasm the nuclei still contained phosphatase. After 2 hours of incubation, the glomeruli in most cases, both in the hydronephrotic as well as in the normal

kidney, showed only faint activity After longer incubation, however, the glomeruli showed considerable activity in most of the slides It seems that in the hydronephrotic kidneys there was a somewhat stronger reaction in the glomeruli and the smaller arterioles This was similar to the previously described findings in choline-deficient kidneys in which the impression was gained that the glomeruli stained more intensely in phosphatase-depleted than in normal kidneys This difference may, however, be only apparent and due to the visual contrast between the strong staining reaction in the glomeruli and the weak reaction in the depleted tubules The young fibroblasts found in two cases in the kidney pelvis revealed an intense phosphatase activity The intense phosphatase activity of young fibroblasts in the rat has been described by Fell and Danielli (29)

COMMENT

In agreement with Gomori (13) the lipase activity in tissue sections was found to be most pronounced in the rat kidney and less in that of the dog and guinea pig, as well as in that of the hamster, rabbit, and mouse Lipase activity was found only in the proximal convoluted tubules of the cortex in all the animal species examined and very occasionally in the epithelium of the kidney pelvis

A somewhat different distribution has been found by Weil and Jennings (30) These investigators used the technique of Linderstrøm-Lang (31) and Glick's (32) micromethod for the determination of lipase (kidney esterase) activity in the rabbit kidney Linderstrøm-Lang's technique consists essentially in estimating the enzymatic activity microchemically and correlating the results statistically with the cells present in alternate microscopic sections Weil and Jennings found the maximum lipase activity in the cortex but also some activity in the medulla The cells of the proximal and distal convoluted tubules were about twice as active as the cells of Henle's loops and about 4 times as active as cells composing the collecting tubules

Lipase activity has been demonstrated by chemical methods not only in liver and pancreas but also in various other organs including the kidneys of various animals and man (33–42) Different substrates have been used for estimating the enzymatic activity The degree of this activity depends, among other factors, to a large extent upon the substrate used (37) Crandall and Cherry (39), for instance, using olive oil as substrate, found only traces of activity in the dog kidney, but fairly strong activity with ethylbutyrate Rona and Lasnitzki (36), using tributyrin, found stronger activity in the guinea pig than in the rat kidney While, in agreement with Gomori, no lipase activity in tissue sections could be demonstrated in the rat spleen with sorbitan monopalmitate, such activity has been demonstrated by chemical estimation with various other substrates (37)

Lipase activity is still present in many of the necrotic cells of kidneys severely

damaged by the application of uranium nitrate and mercuric chloride. As the cells are shed off into the lumen, the tubules become depleted of the enzyme. The regenerating and atrophic epithelium in kidneys of animals surviving the acute intoxication, shows marked depletion in lipase activity Similar depletion is seen in the acute and subacute phase of choline deficiency as well as in the epithelium of the dilated and collapsed convoluted tubules of the hydronephrotic kidneys The changes in phosphatase activity are of an essentially identical nature Both enzymatic systems, therefore, are changed by the experimental damage in a similar manner

The decrease in enzymatic activity as soon as 10 days after ligation of the ureter, is especially remarkable in view of Suzuki's (18) findings after injection of carmine or trypan blue These dyes are excreted by the glomeruli and are partially absorbed by the epithelium of the proximal convoluted tubules where they are concentrated in particulate form (43) Suzuki found, even after many weeks of ligation of the ureter, concentrations of dye in the cells of the proximal convoluted tubules, although these cells were changed morphologically On the other hand, MacNider (44 *a,b*) has shown that after damage with uranium nitrate, the regenerated epithelial tissue of the kidney as well as that of the liver is not only of a changed morphological type but is also resistant to the same, as well as to different toxic substances

Calcification is frequently seen in necrotic cells still containing phosphatase after poisoning with mercuric chloride and uranium nitrate. In contrast, Hepler and Simmonds (22) maintained that calcium is deposited only in tubules in which the epithelium has been so severely damaged that the phosphatase has been rendered inactive, and concluded that phosphatase does not play any part in the process of calcification The findings reported here are in agreement with Gomori's experience (16, 45) that calcification of recently necrosed tissue is dependent upon the presence of alkaline phosphatase.

While the abundance of alkaline phosphatase has been explained by the importance of this enzyme for the reabsorption of sugar from the glomerular filtrate (10, 46), apparently no special function has thus far been inferred from the presence of lipase in the kidney However, certain clinical and experimental facts point to an essential rôle of the kidney in fat metabolism Cholesterol, lipid phosphorus, as well as neutral fat, are frequently elevated in the serum of patients with renal disease (47) Increase in serum lipids has been reported in rats after partial nephrectomy (48, 49), in acute nephritis induced by anti kidney serum (50), and after poisoning with mercury bichloride (51, 52) A *rise in serum lipids has been observed in dogs, after unilateral nephrectomy* (51–53), bilateral nephrectomy (51–54) bilateral ureteral ligation (54), and after poisoning with potassium dichromate, uranium nitrate, and mercury bichloride (51–53) Rise in serum lipids after nephrectomy has also been noticed in cats (55) and monkeys (54) Furthermore a considerable turn

these two agents is indicated by the fact that meningopneumonitis virus immunized mice against both of them Extensive studies on the relationship of psittacosis to other viruses were not attempted because similar investigations are being conducted in other laboratories

Since the immune sera used in the complement fixation tests were produced by immunizing animals with infected tissues of the corresponding species, heterophile antibodies of normal tissues could not be concerned in the observed cross-reactions The group specific complement-fixing antibodies in the sera of animals immunized with the virus of lymphogranuloma venereum probably resulted from an immune response either to an antigenic constituent of the virus particle or to an antigenic product of the interaction of virus and infected tissue The corresponding group specific factor of the meningopneumonitis strains MP-Cal 10, MP-F97, and S-F apparently stimulated less antibody response because sera of mice and guinea pigs immunized with these strains, although showing a high titer with the meningopneumonitis virus, gave slight or no fixation with the virus of lymphogranuloma venereum (Table V) On the other hand, human sera from cases of pneumonitis often gave strong fixation with both antigens from lymphogranuloma venereum and meningopneumonitis (5) These differences in species response cannot be adequately explained on the basis of present observations

Much additional work on differences in pathogenicity will be necessary before a definite classification of these viruses is possible, but a few basic criteria may be noted Only one member of the group under consideration, psittacosis virus, is highly virulent for mice by the intraperitoneal route The virus of meningopneumonitis kills mice only after intraperitoneal injection of massive doses Strains of the agent of lymphogranuloma venereum were much less virulent by any route for mice than the other viruses Differences in virulence for guinea pigs, hamsters, birds, and chick embryos have also been noted Pinkerton (14) has observed that meningopneumonitis virus produces a fatal infection in pigeons after intracerebral inoculation while one strain of psittacosis virus does not All of the strains studied except LGV-LC and LGV-ST produced paralysis of the hind legs of mice after an incubation period of 5 to 6 days following intracerebral injection and some caused paralysis irregularly after intraperitoneal inoculation

The observations recorded in this paper suggest that the viruses causing certain forms of atypical pneumonia and lymphogranuloma venereum in human beings, psittacosis in birds, and other viruses which may have come from mice or ferrets should be classified together as representatives of a group of agents which in various modified forms are distributed widely in nature and cause infections of diverse character The members of this group of viruses are characterized by the formation of elementary bodies which stain with basic aniline dyes, by the possession of antigenic components common to each, and by the

production of meningitis, pneumonia, and granulomatous infiltrations of the skin in experimental animals. Analogous groups of similar size are the *Rickettsiae*, and the pleuropneumonia like organisms It is also apparent that criteria previously used for classification of these agents, particularly with regard to the psittacosis group, have not been adequate

SUMMARY

Animals recovered from infection with the viruses of lymphogranuloma venereum, meningopneumonitis, and psittacosis, were reinoculated in cross-immunity tests with these viruses

In mice immunized by intracerebral or intranasal inoculation a reciprocal partial cross-immunity between the viruses of lymphogranuloma venereum and meningopneumonitis was demonstrated In preliminary experiments, similar cross-immunity between the agents of lymphogranuloma venereum and psittacosis was not definitely demonstrated

Hamsters, white rats, and kangaroo rats recovered from intracerebral or intranasal infection with the virus of lymphogranuloma venereum were more resistant than normal controls to inoculation with the virus of meningopneumonitis

Sera of animals immunized with the viruses of lymphogranuloma venereum and meningopneumonitis showed cross-reactions by complement fixation with antigens of these viruses.

The results indicate an antigenic relationship between the viruses of lymphogranuloma venereum and meningopneumonitis

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A FURTHER STUDY OF THE CROSS REACTION BETWEEN THE SPECIFIC POLYSACCHARIDES OF TYPES III AND VIII PNEUMOCOCCI IN HORSE ANTISERA*

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A constantly increasing number of immunological cross reactions are being studied with the aid of the quantitative precipitin and quantitative agglutinin techniques, and knowledge of this phase of immunological specificity is consequently taking on a more precise character. The present contribution to this subject continues earlier work (1) on the pneumococcus Type III Type VIII cross reaction.

It was shown in the earlier studies that the reaction between the specific polysaccharide of Type VIII pneumococcus (S VIII) and an antipneumococcus (anti Pn) Type VIII horse serum, in the antibody excess region and through the equivalence zone, could be represented by the equation

$$\text{mg. antibody N precipitated} = 2RS - \frac{R^2S^2}{A},$$

in which S = S VIII precipitated, A = antibody nitrogen precipitated at a reference point in the equivalence zone, and R = ratio of A to S at the reference point (*cf.* 1, 2), an equation which may be derived from the law of mass action and which has been shown to be applicable in many immune systems (3). However, the cross reaction between the specific polysaccharide of Type III pneumococcus (S III) and Type VIII antiserum was of a distinctly different character. When S III was plotted against antibody N the resulting curve was not characteristic of the above equation but was composed of a steep initial portion, followed by a less steep, linear section. Since cross reactions are not always similar in the reciprocal sense it was thought of interest to make a quantitative study of the S VIII-anti-S III reaction, and data on this are included in the present report. An attempt was also made to isolate the antibody reactive in linear fashion in the cross reactions, as it was thought that this might show a simpler behavior toward polysaccharide than the antibody as a whole. Finally, experiments were run at 37°C. as well as at 0° in order to test the effect of temperature on the cross reaction.

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EXPERIMENTAL

Materials and Methods—Type III and Type VIII antipneumococcus horse sera[1] were used after absorption with crude Type I pneumococcus "C" substance The Type III and Type VIII pneumococcus polysaccharides (designated S III and S VIII) were prepared according to references 1 and 4

TABLE I

Antibody N Precipitated from Type III Antiserum H 792 by Varying Amounts of S III and S VIII

Amount of S used	Antibody N precipitated by S III from 1 0 ml serum	Ratio antibody N to S III in precipitate	Tests on supernatants	Antibody N precipitated by S VIII from 5 0 ml serum	Ratio antibody N to S VIII in precipitate	Tests on supernatants	Antibody N precipitated by S III from 1.5 ml serum dilution freed from cross reactive antibody	Ratio antibody N precipitated to S III in precipitate	Tests on supernatants
mg	*mg*			*mg*			*mg*		
0 020	0 396	19 8	Excess A*	0 250	12 5	Excess A	0 394‡	19 7	Excess A
0 030	0 538	17 9	" "	0 336	11 2	" "	0 514	17 1	" "
0 050	0 664	13 3	" "	0 454	9 1	" "	0 620	12 4	" "
0 075	0 702	9 4	No A or S	0 534	7 1	" "	0 624	8 3	No A or S
0 10	0 700	7 0	" " " "	0 556	5 6	" "	0 614	6 1	Trace S
0 15	0 726	4 9§	Excess S	0 690	(4 6)	" "	0 654	4 5§	Excess S
0 20	0 748	3 9§	" "	0 756	3 9§	A and S	0 658	3 7§	" "
0 25				0 764		" " "			
0 30				0 816	2 9§	" " "	0 646	3 3§	" "
0 40				0 844‡	2 5§	Excess S			
0 50				0 884	2 5§	" "			
0 75				0 952	2 7§	" "			
1 00				0 950		" "			
Equation‖ mg N precipitated = 23 4 S − 137 S²							Equation‖ mg N precipitated = 23.9 S − 143 S²		

* A = antibody

‡ Single analysis only

§ Amount S in supernatant determined (6 *b, c*)

‖ Calculated to 1 0 mg A N as follows in the experimentally determined linear equation, $\frac{A\,N}{S}$ in precipitate $= 2R - \frac{R^2}{A} S$, A is put = 1 0, changing only the slope of the line

The precipitin determinations were carried out in the usual way (5, 6) by addition of increasing amounts of polysaccharide to accurately measured volumes of serum After 2 days at 0°C the specific precipitates were centrifuged, washed twice in the cold, and analyzed for nitrogen by the micro-Kjeldahl method. All analyses were run in duplicate

Experiment 1 Homologous and Cross Reactions of Type III Antiserum H 792 (Bleeding 2/3/37)—Data are given in Table I and Fig 1 on the addition of increasing

[1] These sera were obtained through the kindness of Dr Ralph S Muckenfuss and the late Dr William H Park of the New York City Department of Health Research Laboratories

amounts of S III and S VIII to the antiserum The equations, calculated to the same antibody content, are seen to be identical before and after heterologous absorption, as in a similar experiment with Type VIII serum (1) The cross reaction, also, was

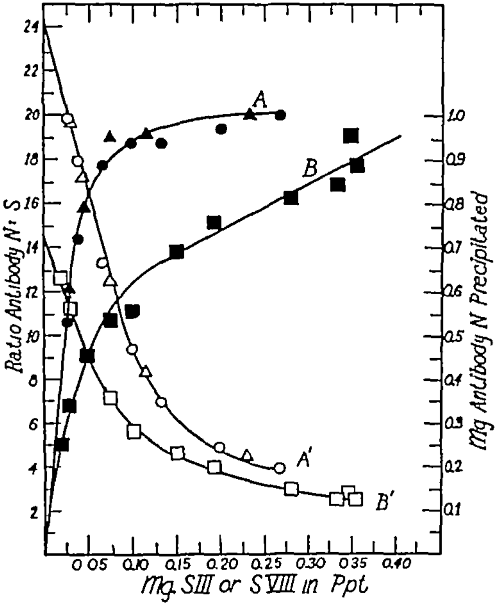

FIG 1 Reaction of antipneumococcus Type III serum H 792 with S III and S VIII. Curves A and A' are for the homologous reaction, B and B' for the cross reaction A' and B' are the respective A N S ratio plots. Circles give the points obtained with the original serum and triangles those after removal of antibody reactive with S VIII. Not all points in Table I are included in the plot.

similar to that for the antipneumococcus Type VIII-S III reaction (1), consisting of a steep initial segment and a less steep, nearly straight portion slope 1 1, along which there was considerable scattering of experimental points. Upon addition of larger amounts of S, the combining ratio of antibody N to S reached a lower limit showing that the precipitate attained constant composition The large factors by which it was necessary to multiply the analytical data for the terminal portion of the curve rendered the slope of this segment uncertain

TABLE II

Comparison of Total Antibody (3 0 Ml Serum, 1 1) and Antibody Fractions in Cross Reaction at 0°C between S III and Anti-Pn VIII Horse Serum 644

S III added	S III in precipitate		Antibody N precipitated	Ratio anti body N to S III in precipitate based on supernatant analyzed with H 644	Supernatants		
	By H 644*	By H 792‡			+ S III	+ Anti Pn III horse serum	+ H 644
mg	*mg*	*mg*	*mg*				
0 017$_8$	Total		0 344	19 5	+++		
0 031$_3$	"		0 500	16 0			
0 047	"		0 638	13 6	+	±	–
0 071	"		0 706	9 9	+	±	–
0 113	0 110	0 110	0 786	7 1	–		
0 188	0 176	0 174	0 940	5 3	–		
0 245	0 220	0 213	1 04	4 7			
0 261	0 229	0 223	1 10	4 8			
0 353§	0 273	0 258	1 12	4 1			
0 423§	0 273	0 273	1 13	4 1			
0 522	0 30	0 29	1 15	3 8			
Antibody N Precipitated per 6 0 Ml of H 644 A Solution by Varying Amounts of S III							
0 017$_7$	Total		0 314	17 7	+++		–‖
0 037$_6$**	"		0 514	13 7	–		–
0 062$_6$‡‡	"		0 572	9 1	–		–
0 106§	0 100		0 600	6 0	–		+

Equation Antibody N precipitated = 21 S – 90 S², calculated values for A at the first 3 points are 0 312, 0 521, and 0 569, respectively

Antibody N Precipitated per 6 0 Ml of H 644 B by Varying Amounts of S III							
0 060$_4$§§	0 057		0 334	5 9			
0 113**	0 095		0 430	4 5			
0 176‡‡	0 128		0 495	3 9			
0 282‖‖	0 156		0 516	3 3			
0 470‖‖	0 214		0 576	2 7			

* Supernatant analyses in region of excess S III, only, carried out with anti-Pn VIII serum H 644 and quantity found deducted from amount originally taken

‡ Supernatant analyses in region of excess S III, only, carried out with anti Pn III serum H 792

§ 2 0 ml. of serum actually used for analyses, with corresponding amount of S III

‖ Supernatants in this series were tested with H 644 A

** 5 0 ml of serum actually used, with corresponding amount of S III

‡‡ 4 0 " " " " " " " " " "

§§ 7 0 " " " " " " " " " "

‖‖ 3 0 " " " " " " " " " "

Experiment 2 Fractionation of Antipneumococcus Type VIII Horse Serum H 644, (1/5/37) —The equation describing the homologous reaction of this serum was

$$\text{mg N precipitated} = 21.5\ S - 108\ S^2\ A\ N = 1.07\ mg.,$$

practically the same as that of a bleeding from the same horse taken one year earlier (1) The cross reaction data on this serum are given in Table II and Fig 2 In this system, as in the S VIII-anti-S III reaction, the combining ratio of antibody to poly saccharide approached a constant value. The course of the reaction curve, A, in Fig 2, was similar to that of the earlier bleeding (1) The slope of the linear portion, 2 4, was practically the same as that, 2.5 found in (1)

To 50 ml of serum H 644 a quantity of S III was added corresponding to a point at the beginning of the linear portion of the curve in order to separate, if possible, the

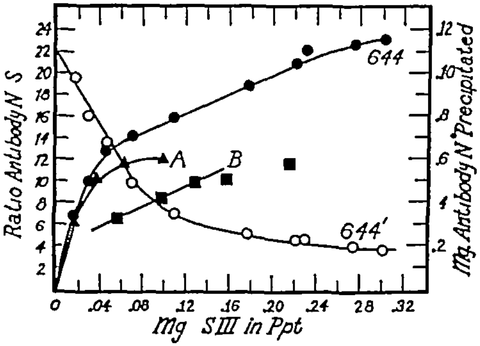

FIG 2 Reaction of antipneumococcus Type VIII serum H 644 with S III.

antibodies characteristic of the different parts of the reaction plot. The specific precipitate obtained was dissociated by Liu and Wu's modification (7) of Felton's (8) dilute alkali method, and a series of precipitin analyses was carried out with the recovered antibody solution (644 A) and S III. The curve resembled that of the first portion of the cross reaction plot. The supernatant from the original precipitation, designated 644 B, was also analyzed by the quantitative precipitin method. The slope of the 644 B line was 2.2 or almost the same as that of the original serum The results are given in Table II and plotted in Fig. 2

Antipneumococcus Type VIII horse sera bled from horse 909 in 1937 and in 1939 were employed for further repetitions of the cross reaction experiments. The data are omitted since they closely resembled those given in Table II and Fig 2 The slope of the linear portion of the curve for the 1937 bleeding was 2 9 and that for the 1939 bleeding was 2.8 The antibody S III ratios also approached a lower limiting value as in the case of serum 644

Experiment 3 Cross Precipitation at 37°C —Data obtained at 37°C. on sera H 644

and H 909 (1939 bleeding) are given in Table III The tubes were incubated for 0 5 hour, centrifuged at 37°C, and washed twice with saline in the cold The supernatants were chilled and allowed to stand overnight in the ice box Additional precipitate appeared in the supernatants which contained excess antigen The sum of the antibody N precipitated at 37°C and that deposited after chilling did not equal the quantity precipitated in analyses run at 0°C throughout (*cf* also (6*a*))

Quantitative analyses in the two cross reacting systems showed a much greater error near the antibody maximum when run at 0° than at 37°C Even with strict temperature control successive analyses often differed by as much as 0 05 mg of N This tendency seemed greater in certain sera than in others, and although the reason for it is not known, it recalls Goodner and Horsfall's observation (9) of coprecipitation

TABLE III

Antibody N Precipitated at 37°C from 3 0 Ml (1 1) Type VIII Antiserum H 644 by Varying Amounts of S III

Amount of S III added	Antibody N precipitated	Ratio antibody N to S III	Additional antibody N precipitated by subsequent chilling to 0°C	Tests on supernatants
mg	*mg*		*mg*	
0 020	0 250	12 5	0 003	Excess A
0 030	0 324	10 8	0 003	" "
0 050	0 424	8 3	0 003	" "
0 075	0 530		0 003	A and S
0 10	0 584		0 003	" " "
0 15	0 668		0 021	" " "
0 20	0 734		0 047	" " "
2 0 Ml Type VIII Antiserum 909				
0 127	0 504		0 084	Excess S
0 177*	0 528		0 070	" "

* Three-quarters of this amount and 1.5 ml antiserum were actually used

of Types I and II antibodies from polyvalent and mixed antipneumococcus horse sera at 0° while more strictly specific precipitation occurred at 37°C

DISCUSSION

The quantitative theory of the precipitin reaction (2) involved the assumption that the numerous antibodies in antisera might be treated mathematically as a single substance of average reactivity, multivalent with respect to antigen, which, in turn, was multivalent with respect to antibody In this way the aggregation and the multiple combining proportions characteristic of immune reactions such as the precipitin and agglutinin reactions could be quantitatively accounted for (3) It was, however, realized that antisera actually contain a complex mixture of antibodies differing possibly both in the number and kinds of groupings reactive with the antigen Numerous instances were given (3) in which partial absorption of antisera with homologous antigen left behind antibodies different in reactivity from the assumed average in the original serum and characterized by a different equation

In the first paper on the Type III Type VIII cross reaction, however, it was shown that cross absorption did not result in an analogous fractionation of the antibodies with respect to their reactivity toward homologous polysaccharide (1) Although about one third of the antibodies in a Type VIII antipneumococcus horse serum could be precipitated by S III, the precipitin reaction between S VIII and the Type VIII anticarbohydrate followed exactly the same course and gave the same equation, when calculated to the same antibody content, whether carried out in whole serum or in serum deprived of the considerable proportion of antibody cross reactive with S III This has since been confirmed in parallel studies on other Type VIII antisera It will be noted from Table I and Fig 1 that a similar state of affairs obtains in Type III antiserum, the homologous reaction and equation, recalculated to the same amount of antibody, remaining the same after removal of the antibodies precipitated by S VIII This may be taken to indicate a random distribution, among the antibodies of different reactivities, of groupings or configurations capable of reacting with heterologous specific polysaccharide

While the homologous reaction pairs, S III anti S III and S VIII-anti-S VIII yielded curves of the type previously observed in horse sera (1, 2), the heterologous pairs, S III anti S VIII (1) and S VIII-anti S III followed a different but mutually similar course After an initial steep, curving portion in the plots (Figs 1 and 2) resembling the homologous reactions there was a relatively sharp transition into a less steep, linear portion suggestive of the reaction plot found by Scherp for Type I meningococcus specific polysaccharide in polyvalent antimeningococcus horse sera (10), and in exaggerated measure, conforming to Goodner's presentation (11) of even homologous reaction curves as a series of small linear segments Even along the linear portion of the cross reaction curve the composition of the specific precipitate varied, but tended to become constant at lower antibody N to S ratios as the amount of specific polysaccharide was increased With 10 times the quantity of S giving maximum cross precipitation partial inhibition ensued in the S III-anti S VIII reaction while in the S VIII anti S III system precipitation was merely delayed, *not diminished* *A further 5-fold increase in S resulted in complete* inhibition of precipitation in the former system and nearly complete inhibition in the latter In both reactions, then, soluble compounds are possible with lower antibody N S ratios than the final constant ratio insoluble compound, just as in homologous precipitin reactions (*cf* also (12))

It was noted that removal of the cross reacting antibodies corresponding to the initial curved segment of the plot resulted in the failure of about one-half of the remaining cross reacting antibody to precipitate with S III. A similar effect has been observed in the serial precipitation of many antisera by small amounts of antigen and has been interpreted on the assumption that the non precipitable portion of antibody is univalent with respect to antigen and can take part in aggregate formation only in the presence of sufficient multivalent

antibody (3, 13) On this basis, then, the entire linear portion of the cross reaction curve would be due to the addition of univalent (and by itself non-precipitable) antibody to S III-A aggregates formed with the multivalent antibody of the initial segment of the curve In the attempted separation of the antibody into portions characteristic of the two segments it would seem that sufficient multivalent antibody remained with the linear portion, 644 B, to permit precipitation by S III of roughly one-half of the total cross reacting antibody This is borne out by the failure of the line B in Fig 2 to pass through the origin The interpretation given also accounts for the drift in composition noted in Table II

If one considers the linear segment as a separate curve, apart from the remainder of the plot, it is found that the quantity of antibody precipitated is directly proportional to the amount of S III added Since combining proportions of antigen and antibody remain constant over this range, it is possible to calculate an immunological equivalent weight for S III in the cross reaction The proportionality constant of antibody N to antigen, or the slope, equals 2 5 for serum H 644, multiplied by 6 3 this yields a protein polysaccharide weight ratio of about 16 If 1,000,000 be taken as the molecular weight of horse antibody to pneumococcus (14), and its valence with respect to S III equal to 1, as discussed above, the equivalent weight of S III is found to be 62,000, which equals about 180 glucuronoglucose units This would represent a minimum value for the molecular weight of S III A similar calculation for S VIII, based on Experiment 1, leads to the value 140,000

Of the numerous instances of cross reactivity studied some have been found to be reciprocal and others unilateral, but criteria are lacking by which predictions can be made as to which behavior a given cross reaction will show The specificities and cross reactivities of azoantigens containing sugars and sugar acids as determinant groups have been studied by Avery and Goebel (15) and by Goebel (16) and have been related to the chemical structure and spatial relationship of the sugar haptens employed The present study permits a correlation between the chemical constitution of two naturally occurring polysaccharides, S III and S VIII, and the quantitative behavior of the reciprocally similar cross reactions in both directions between these polysaccharides and horse antisera to Types III and VIII pneumococci

Since S III (17) and S VIII (18) contain the same aldobionic acid as a structural unit, a measure of cross reactivity is to be expected Evidence has been given, however, that the serologically reactive unit is a larger portion of the polysaccharide molecule than a single chemical structural unit (19, 1) While S III is a polymer of the aldobionic acid unit, S VIII contains, in addition, approximately two glucose molecules for every aldobionic acid residue (1, 18) Cross precipitation in either the S III-anti-S VIII system or the S VIII-anti-S III system would therefore involve only that fraction of the antibodies carrying reactive groupings of suitable configuration (*cf* also Hooker and Boyd (20))

An appreciable portion of the cross reacting fraction would be expected to contain more than one reactive grouping per antibody molecule. It might also be predicted from a knowledge of the structures of S III and S VIII (as yet complete only for S III (21)), that, weight for weight, the polyaldobionic acid S III would be a more efficient cross precipitant for anti-S VIII than S VIII for anti-S III This would follow, because in S VIII the common aldobionic acid unit, to a multiple of which the cross reactivity is ascribed, comprises only about 60 per cent of the molecule whereas S III is wholly a polyaldobionic acid That this prediction is borne out is shown by the slopes of the linear portions of the cross reaction curves In the S III anti-S VIII system these slopes are more than twice as great as that of the single S VIII-anti S III system studied, so that along this portion of the curve more S VIII is required to precipitate a given weight of anti S III than is necessary in the case of S III to precipitate the same weight of anti S VIII. This not only holds for the linear segments of the curves, but also for the initial steep portions, as may readily be determined either from the intercepts of the line connecting the antibody N S ratios of the first points in the curves, or by noting on Figs 1 and 2 the amounts of antibody N brought down in the cross reactions by 0 01, 0 02, and 0 03 mg of S III and S VIII, respectively

Since the cross reactivities of S III and S VIII seem reciprocally equal to the proportion of common aldobionic (cellobiuronic) acid contained in each poly saccharide, the cross reactions being of the same type, but with numerical differences of the order expected from as much of the chemical structures as is known, a further, admittedly speculative, deduction might be drawn

If one writes the structure of S III in conformity with (21) but schematically as

$$(a)\quad \left\{\mathrm{Gn\ Gl}\right\}\left\{\mathrm{Gn\ Gl}\right\}\left\{\mathrm{Gn\ Gl}\right\}\left\{\mathrm{Gn\ Gl}\right\}\quad ,$$

in which Gn = glucuronic acid and Gl = glucose residues, and brackets indicate cellobiuronic acid residues, a number of alternative formulas for S VIII containing the same unit suggest themselves

$$(b)\quad \left\{\mathrm{Gn\ Gl}\right\}\ \mathrm{Gl\ Gl}\ \left\{\mathrm{Gn\ Gl}\right\}\ \mathrm{Gl\ Gl}\ \left\{\mathrm{Gn\ Gl}\right\}\ \mathrm{Gl\ Gl}\quad ,$$

$$(c)\quad \left\{\mathrm{Gn\ Gl}\right\}\begin{matrix}\mathrm{Gl}\\ \mathrm{Gl}\end{matrix}\left\{\mathrm{Gn\ Gl}\right\}\begin{matrix}\mathrm{Gl}\\ \mathrm{Gl}\end{matrix}\left\{\mathrm{Gn\ Gl}\right\}\begin{matrix}\mathrm{Gl}\\ \mathrm{Gl}\end{matrix}$$

$$(d)\quad \left\{\mathrm{Gn}\ \begin{matrix}\mathrm{Gl}\\ \mathrm{Gl}\\ \mathrm{Gl}\end{matrix}\right\}\left\{\mathrm{Gn}\ \begin{matrix}\mathrm{Gl}\\ \mathrm{Gl}\\ \mathrm{Gl}\end{matrix}\right\}\left\{\mathrm{Gn}\ \begin{matrix}\mathrm{Gl}\\ \mathrm{Gl}\\ \mathrm{Gl}\end{matrix}\right\}$$

In (*c*) and (*d*) the side-chain glucose residues are arbitrarily placed, if they were shifted, or placed in tandem in (*d*) the structural type would remain much the same The great similarity of the cross reactions would seem to require as great a similarity in the structure of the two polysaccharides as possible (*a*) and (*d*) have the same polyaldobionic acid chain, so that (*d*) or one of its alternative forms,

$$\left\{\begin{matrix} \text{Gn} & \text{Gl} \\ \text{Gl} & \text{Gl} \end{matrix}\right\}_x \quad \text{or} \quad \left\{\begin{matrix} & \text{Gl} \\ \text{Gn} & \text{Gl} \\ \text{Gl} & \end{matrix}\right\}_x \quad \text{or} \quad \left\{\begin{matrix} \text{Gl} & \\ \text{Gl} & \\ \text{Gn} & \text{Gl} \end{matrix}\right\}_x \quad \text{or} \quad \left\{\begin{matrix} & \text{Gl} \\ & \text{Gl} \\ \cdot\text{Gn} & \text{Gl} \end{matrix}\right\}_x$$

would be a distinctly probable structure, if serological data alone are considered. But unless S III and S VIII have greatly different molecular weights this type of structure seems less probable than one of the linear forms since the viscosities of the two polysaccharides in undegraded form (4, 1) are not very different If one compares (*b*) and (*c*) with (*a*) and assumes similar linkages and spatial arrangements it becomes evident that in (*b*) every cellobiuronic acid unit would correspond spatially with alternate cellobiuronic acid units in (*a*), while (*c*) would show a periodicity of this kind only between alternate units in (*c*) and every third unit in (*a*) Therefore, taking all available chemical, physical, and quantitative serological evidence into consideration, (*b*) would seem the most probable structure for S VIII although (*d*) and (*c*) cannot, of course, be excluded A definite answer must await methylation studies such as have led to the elucidation of the structure of S III

Another rather unexpected by-product of the quantitative study of these cross reactions emerges from a consideration of the initial steep section of the curve in the S III-anti-S VIII reaction In the equation of the line connecting the first four antibody N S ratio points on curve 644′, Fig 2, the slope, $-R^2/A = 171$, and 2R, the intercept on the ordinate, = 22 A, therefore, = 0 7 Since only the slope is affected in putting A = 1 0, the equation of the curve, obtained by multiplying both sides of the linear equation by S, becomes

$$\text{mg antibody N precipitated} = 22\ S - 120\ S^2, \text{ at A} = 1\ 0$$

The equation of the homologous S VIII-anti-S VIII reaction calculated to the 1 0 mg A level is

$$\text{mg antibody N precipitated} = 21\ 5\ S - 116\ S^2$$

or practically identical with that of the cross reaction Unfortunately the data given in (1) cannot be used for an additional test of this relationship since only one point on this part of the curve was determined However, the dissociated antibody (644 A) from the fractionation of serum 644 (Table II, and Fig 2, curve A) also has the same equation,

$$\text{mg antibody N precipitated} = 21\ S - 114\ S^2,$$

as that of the homologous reaction when calculated to 1 0 mg of cross reacting antibody N The virtual identity of the equations indicates that an appreci-

able fraction of the antibody reacts in exactly the same way with S III and with S VIII As far as can be determined by the sensitive quantitative method, this fraction of the antibody fails to distinguish between the two polysaccharides This serves again to emphasize both the close structural relation between the two polysaccharides as well as the sharp differences in reactivity between different fractions of the antibodies elicited in an animal by immunization with a single antigen (*cf* also (3)) These results also extend an earlier observation that the antibody dissociated from a specific precipitate of S III and anti S VIII was entirely precipitable by either polysaccharide (22)

A corresponding antibody fraction with identical reactivity toward S III and S VIII was not found in the antipneumococcus Type III serum studied (Table I and Fig 1) It will be noted that the equation of the initial segment of the cross reaction curve, calculated to 1 0 mg of cross reacting antibody N in the same way as in the preceding instance,

$$\text{mg antibody N precipitated} = 14.5\,S - 52\,6\,S^2$$

is distinctly different from that of the homologous reaction,

$$\text{mg antibody N precipitated} = 23\,4\,S - 137\,S^2$$

at the same antibody N level The failure of the two cross reactions to show strictly reciprocal behavior in this sense, while not predictable on the basis of available information, is at least in accord with the points stressed, since, as noted above, S VIII is only in part a polycellobiuronic acid such as S III

Thus the S III anti S VIII and S VIII anti-S III cross reactions show, in general, a reciprocal character that might have been expected from the close structural similarity of the specific polysaccharides comprising the hapten portion of the distinctive antigens of the two pneumococcus types Quantitative analysis of the cross reactions, has, however, brought to light distinct differences in the course of the two reactions and permitted a correlation between these differences and the known chemical structures of the two polysaccharides

It is also evident that at least three distinct kinds of anticarbohydrate[2] are evoked in horses in response to the stimulus of a type specific pneumococcus antigen such as, for example, that of Type VIII. Two of these forms make up the cross reactive fraction, which usually comprises one-quarter to one third of the total As already noted, this portion is completely precipitable by S III or by S VIII It may be fractionated by means of the cross reacting polysaccharide, S III, into (1) a portion characterized by a sharply ascending reaction curve, and (2) a fraction showing a linear segment and ultimately constant composition of the specific precipitate This, if it could be obtained entirely separate from the other fraction, would appear to be univalent with

[2] Antibodies to the somatic C-substance need not be considered here, since they were removed from all antisera in advance.

respect to S III, although not with respect to the homologous S VIII Finally, after separation of the cross precipitable antibody from the antiserum, the principal antibody fraction (3), at least two-thirds of the total, is found to be rigidly type specific in that cross precipitation between Types III and VIII does not occur Even this antibody is not homogenous, but is composed of fractions of varying reactivity toward the homologous polysaccharide as shown by the identity of its characteristic equation with that of the antiserum as a whole The concept "antibody" would therefore seem to refer, not to serum globulin modified in a single manner, but to a series of modified globulins separable and identifiable as distinct fractions and limited in number mainly by the cross reactivities and quantitative criteria available for their characterization These complexities are, however, far from hopeless, as they may be accounted for even quantitatively on the basis of varying numbers and kinds of reactive molecular groupings, for example, as in the present communication and in earlier papers from this laboratory

SUMMARY

1 The cross reaction of the specific polysaccharide of Type VIII pneumococcus with Type III antipneumococcus horse serum has been studied quantitatively and found similar to the S III-anti-S VIII reaction

2 Contrasted with the general similarity of the two-segment reaction curves were distinct qualitative and quantitative differences in the course and character of the reciprocal reactions with respect to each segment

3 These differences could be interpreted in terms of the known chemical differences between the specific polysaccharides of the two types A minimum molecular weight of 62,000 was calculated for S III and 140,000 for S VIII

4 It was also found possible to fractionate the Type VIII antibody into portions characteristic of each segment of the cross reaction curve At least three different kinds of Type III and Type VIII anticarbohydrates were identified

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THE QUANTITATIVE DETERMINATION OF INFLUENZA VIRUS AND ANTIBODIES BY MEANS OF RED CELL AGGLUTINATION

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In a recent brief communication we described the agglutination of chicken red blood cells by materials containing the PR8 and Lee strains of influenza virus (1) Furthermore, it was shown that the addition of specific immune serum inhibited the agglutination in the presence of the homologous virus, but not when heterologous strains were used. This paper presents the results of further experiments related to these phenomena. The first experiments deal primarily with the correlation between the agglutinating capacity of virus suspensions and their infectivity for mice. A greater number of experiments have been directed toward correlating the agglutination inhibiting power of various sera with their virus-neutralizing capacity Experiments primarily concerned with the mechanism of agglutination will be discussed in a later report.

Methods

Preparation of Virus Suspensions—The allantoic fluid of infected chick embryos has been used as the principal source of virus throughout the work reported here. Eleven-day old white Leghorn embryos were used for inoculation The shell was first sterilized with alcohol, and a small puncture was made over the air sac with a blunt dissecting needle A second hole was made over the region where the chorioallantoic vessels were visible. The point of a 23 gauge needle was inserted into the latter hole, and 0 1 cc. of virus suspension was injected just beneath the egg shell into the allantoic sac. Usually infected allantoic fluid, diluted to 10^{-3} or 10^{-6} with saline, was used for the inoculum. The holes were sealed with paraffin and the eggs were incubated at 37°C.

After 48 hours the allantoic fluid was removed from the eggs with a minimum of contamination with embryonic red cells. While this was slightly difficult technically, the increased care was justified by the higher virus titer of the fluid obtained. A moderate number of red cells although quickly removed from the fluid by centrifuga tion, took out 50 to 90 per cent of the virus originally present The eggs were opened by removing the shell over the air sac with forceps, and care was taken not to tear the chorioallantoic membrane. Closed, pointed forceps were used to pierce the exposed membrane near its outer margin The forceps were then allowed to open,

and the membrane was held up like a tent This opened the allantoic sac so that the fluid could be aspirated with a syringe and large bore needle (13 gauge) or with a rubber bulb on a capillary pipette Care was taken not to aspirate any blood which ran down the torn membrane An average of 4 to 5 cc. of cloudy fluid was obtained from each egg, and occasionally as much as 10 cc could be aspirated

The allantoic fluid from several hundred eggs was pooled and then redistributed into small lusteroid tubes These tubes were quickly frozen and stored at $-72°C$ in a CO_2 ice box Under these conditions of storage both the agglutinating capacity and the virus titer as determined in mice have remained essentially unchanged for several months

Strains of Virus Used—Allantoic fluid was prepared in the manner described above using four different strains of influenza virus the mouse-adapted PR8 and the W S strains of influenza A virus, the Lee strain of influenza B virus, and swine influenza virus All except the swine strain had had several passages in eggs prior to use The allantoic fluid containing swine virus was prepared from eggs which had been inoculated with infected mouse lung

Preparation of Chicken Red Cells—Except when otherwise noted, the cells used for all the tests have been a 2 per cent suspension of adult chicken red cells Chickens were bled from a wing vein into a syringe containing 10 cc of 2 per cent sodium citrate The citrated blood was filtered through gauze to remove the small clots, and the cells were washed three times in saline The cells were removed by low-speed centrifugation after each washing After the third washing the packed cells plus two volumes of saline were put into 15 cc graduated centrifuge tubes and spun at 900 R P M. for 8 minutes The sedimented red cells were diluted with saline to fifty times their volume The red cell count on such 2 per cent suspensions was 160,000 to 180,000 per c. mm The chickens were usually bled on the first day of the week, and the red cells were washed and stored at 4°C after packing the final time They were diluted just before use The red cells, when stored in this way, could be preserved in a satisfactory condition for at least a week.

Titration of the Red Cell Agglutinating Capacity of Influenza Virus Suspensions—The test tubes used in all the *in vitro* agglutination experiments were 7 cm long and had an internal diameter of 0 8 cm For agglutination titrations, series of twofold dilutions of the virus suspensions were made in saline To 1 cc of each dilution was added 1 cc. of the 2 per cent red cell suspension The tubes were immediately shaken until the cells were well mixed The titrations then stood at room temperature, without being shaken or disturbed, for 1 hour before reading

Titration of Agglutination-Inhibiting Substances in Sera—The sera to be tested were diluted in twofold steps in saline. To 0 5 cc of each serum dilution was added 0 5 cc of virus suspension, using the same concentration of virus in each tube The virus suspension had previously been diluted to four times the desired final concentration To the mixture of serum and virus was added 1 cc. of a 2 per cent red cell suspension, and the tubes were shaken until the cells were well dispersed The agglutination was read at 1 hour Throughout this paper the concentrations of serum and virus suspension are given in terms of the final concentration, after the red cells have been added In certain inhibition experiments the dilutions of serum were made in normal horse serum or normal ferret serum instead of in saline, so that the total amount of

serum in each tube (normal plus immune) would be the same. All ferret sera were inactivated at 56°C. for 30 minutes before use. The human sera were not inactivated.

Two controls were included in each test a positive control containing red cells, virus suspension, and the same diluent used in making the serum dilutions, and a negative control containing red cells and saline but no virus

Grading the Agglutination Tests —The tests were all read at 1 hour's time and were viewed against a bright white background For this purpose a 15 watt "daylight" fluorescent light proved to be the most useful. The racks were placed directly against the light.

The amount of red cell sedimentation, rather than visible agglutination was taken as the index of the degree of reaction since it was easier to see In the negative control tube the red cells slowly settled during the hour before the test was read. About 3 to 4 mm from the top of the fluid column was a sharp sedimentation boundary above which the saline was clear The cells settling out at the bottom formed a small round, sharply outlined disk, but the density of the lower three fourths of the cell suspension remained unchanged.

In the tubes in which agglutination occurred, the masses of aggregated cells usually settled to the bottom in 1 hour Those cells which remained in the supernatant fluid were usually finely dispersed and not granular, although occasionally clumps adhered to the sides of the tubes. In the tubes in which agglutination was most marked only a thin veil of non granular cells was left in suspension. With decreasing degrees of agglutination the density and cell concentration of the supernatant fluid approached that of the negative control tube. It was the density of the cells remaining in suspension which was used for reading the tests

In order to make the grading of the reaction more objective the density of the cellular suspensions in the various tubes was compared with the density of standard suspensions of red cells in saline These standard suspensions were made from the same red cell preparation used in the tests The tube being read was placed between two tubes of the same standard red cell concentration and the density of the cells in the lower half of the tube was compared with the known dilutions. For the standard suspension, red cells were prepared in concentrations of 1 0 0 75 0.50, and 0.30 per cent. Tubes in which the density fell between that of the 1 0 per cent and the 0 75 per cent standard were called one plus. Those with a density between that of the 0 75 and the 0.50 per cent standards were called two plus, those between 0 50 and 0.30 per cent were graded three plus, and all tubes with a density less than that of the 0.30 per cent suspension were designated four plus.

With the increasing degrees of agglutination the size of the disk in the bottom of the tube also increased In the negative control tube the disk in the bottom was small and sharply outlined When agglutination occurred the margins of the bottom disk often had a characteristic irregular lacy pattern made up of clumps of cells. This was especially true with very slight degrees of agglutination When a tube had this granular pattern on the bottom with no decrease in density of the supernatant suspension it was called plus-minus.

The end point in all agglutination titrations and serum inhibition tests has arbitrarily been taken to be the dilution where two plus agglutination occurs. If two plus agglutination does not occur in any tube, the end point is assumed to be half way

and the membrane was held up like a tent This opened the allantoic sac so that the fluid could be aspirated with a syringe and large bore needle (13 gauge) or with a rubber bulb on a capillary pipette Care was taken not to aspirate any blood which ran down the torn membrane An average of 4 to 5 cc. of cloudy fluid was obtained from each egg, and occasionally as much as 10 cc could be aspirated

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The amount of red cell sedimentation rather than visible agglutination, was taken as the index of the degree of reaction since it was easier to see. In the negative control tube the red cells slowly settled during the hour before the test was read. About 3 to 4 mm from the top of the fluid column was a sharp sedimentation boundary above which the saline was clear The cells settling out at the bottom formed a small, round, sharply outlined disk, but the density of the lower three-fourths of the cell suspension remained unchanged

In the tubes in which agglutination occurred, the masses of aggregated cells usually settled to the bottom in 1 hour Those cells which remained in the supernatant fluid were usually finely dispersed and not granular, although occasionally clumps adhered to the sides of the tubes. In the tubes in which agglutination was most marked only a thin veil of non-granular cells was left in suspension With decreasing degrees of agglutination the density and cell concentration of the supernatant fluid approached that of the negative control tube. It was the density of the cells remaining in suspension which was used for reading the tests.

In order to make the grading of the reaction more objective, the density of the cellular suspensions in the various tubes was compared with the density of standard suspensions of red cells in saline. These standard suspensions were made from the same red cell preparation used in the tests The tube being read was placed between two tubes of the same standard red cell concentration, and the density of the cells in the lower half of the tube was compared with the known dilutions. For the standard suspension red cells were prepared in concentrations of 1 0 0 75, 0 50, and 0.30 per cent. Tubes in which the density fell between that of the 1 0 per cent and the 0 75 per cent standard were called one plus. Those with a density between that of the 0 75 and the 0.50 per cent standards were called two plus, those between 0 50 and 0.30 per cent were graded three plus, and all tubes with a density less than that of the 0.30 per cent suspension were designated four plus.

With the increasing degrees of agglutination the size of the disk in the bottom of the tube also increased. In the negative control tube the disk in the bottom was small and sharply outlined When agglutination occurred the margins of the bottom disk often had a characteristic irregular lacy pattern made up of clumps of cells. This was especially true with very slight degrees of agglutination. When a tube had this granular pattern on the bottom with no decrease in density of the supernatant suspension it was called plus-minus.

The end point in all agglutination titrations and serum inhibition tests has arbitrarily been taken to be the dilution where two plus agglutination occurs. If two plus agglutination does not occur in any tube the end point is assumed to be half way

between two serial dilutions, one showing more and the other less than two plus agglutination

Tests in Mice—The serum neutralization tests in mice were done in the manner described by Horsfall (2) except that twofold instead of fourfold serum dilutions were used The virus titrations in mice were done in the usual way except that dilutions of virus were made in steps of $10^{-0.5}$ instead of 10^{-1} The immune ferret sera used were obtained by bleeding ferrets 14 days after inoculation intranasally with living virus

EXPERIMENTAL

Correlation of Agglutination Titers and 50 Per Cent Mouse Mortality Titers of Suspensions of Influenza Virus—In the first experiment different preparations of the same strain of influenza virus (PR8) were tested by *in vitro* agglutination and by intranasal inoculation of mice in order to compare the titer obtained

Two preparations of infected mouse lung, one of allantoic fluid, and two of ground whole chick embryo, were used Each suspension was centrifuged at low speed until it was clear Some of the preparations were stored for as long as 6 weeks at −72°C, while others were tested immediately after harvesting *In vitro* titrations were set up as described under Methods At the same time mouse titrations were done on these suspensions using dilutions of virus in $10^{-0.5}$ steps

The results, recorded in Table I, show that the mouse lethal titers and the agglutination titers of these suspensions parallel each other over a wide range of virus concentration The figures in the last column of the table represent the concentrations of 50 per cent mouse lethal doses in the *in vitro* end point dilution (++) of the various virus suspensions The relatively slight variation in these concentrations ($10^{3.6}$ to $10^{3.9}$) when virus from very different sources was tested suggests that under these conditions the *in vitro* titration may be a good index of the amount of lethal influenza virus in a suspension

However, it must be emphasized that this correlation holds only when freshly prepared or well preserved virus suspensions are tested, that is to say when all of the virus presumably is pathogenic, for it can be demonstrated that the infectivity of the virus can be destroyed without destroying the capacity of agglutinating red blood cells For example, when infected allantoic fluid was heated at 56°C for 15 minutes, the infectivity of the preparation was completely lost, while the *in vitro* agglutination titer remained undiminished Likewise, when infected allantoic fluid was allowed to stand at room temperature for several days, the mouse lethal titer slowly diminished but the *in vitro* titer remained constant Therefore, to measure the infectivity of a preparation by the *in vitro* test, the suspension must contain no great proportion of inactivated virus

Another exception to the correlation between the two methods of titration is afforded by infected ferret lung A suspension of ground infected ferret lung

TABLE I

Comparison of Agglutination and Mouse Mortality Titrations on Different Preparations of PR8 Virus

Source of virus	Dilution of virus										Saline control	*In vitro* titer	50 per cent mouse mortality titer	Mouse lethal titer/agglutination titer
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560				
Mouse lung 335th passage		++++	++++	++++	++++	++++	++++	+++	++	±	0	1,280	$10^{-6.7}$	$10^{3.6}$
Mouse lung 336th passage		++++	++++	++++	++++	++++	++++	+++	±	0	0	960	$10^{-6.3}$	$10^{3.5}$
Allantoic fluid		++++	++++	++++	++++	++++	++	+	0	0	0	320	$10^{-6.0}$	$10^{3.5}$
Ground whole chick	++++	++++	++	0	0	0	0	0	0	0	0	20	$10^{-5.2}$	$10^{3.9}$
Ground whole chick	++++	+++	±	0	0	0	0	0	0	0	0	15	$10^{-4.9}$	$10^{3.7}$

* Expressed as the number of 50 per cent mouse mortality doses in 0.05 cc. of the dilution of virus suspension which caused two plus agglutination of red cells.

containing a mouse-adapted strain of PR8 virus at a high titer would not agglutinate red cells in any dilution. That this may be due to inhibitory substances in the ferret lung is shown by the fact that when ground normal ferret lung is added to infected allantoic fluid, red cell agglutination is inhibited.

While the experiment summarized in Table I shows a certain consistent relationship between mouse lethal titer and agglutination titer when different preparations of the same strain are tested, an experiment recorded in Table II shows the relationship between these two titers when different strains of influenza virus in allantoic fluid are compared. It will be seen that four different strains, some of them antigenically unrelated, showed a remarkable constancy in their *in vitro* titer, differing by not more than twofold. Simultaneous mouse titrations on the same preparations, however, demonstrated widely differing capacities to kill mice. For example, the W.S. strain, which in this experiment has

TABLE II

Comparison of Agglutination and Mouse Mortality Titrations on Four Different Strains of Influenza Virus

Virus strain	Dilution of virus						Agglutination titer	50 per cent mortality titer
	1:32	1:64	1:128	1:256	1:512	1:1024		
PR8	++++	++++	+++	++	±	0	256	$10^{-6.0}$
W.S.	++++	++++	++	0	0	0	128	$10^{-6.5}$
Swine	++++	++++	++	0	0	0	128	$10^{-4.2}$
Lee	++++	++++	+++	++	0	0	256	$10^{-4.0}$

the highest mortality titer in mice, gives a lower *in vitro* titer than the Lee strain, which has the lowest mortality end point in mice.

It is clear from this experiment that the agglutinating capacity of virus suspensions and their ability to cause lesions or death are independent variables when different virus strains are compared. In view of their wide variation in this latter respect, it was surprising that the allantoic fluid preparations of all the strains so far studied showed such a constant agglutination titer.

Measurement of Agglutination-Inhibiting Substances in Serum.—In a previous paper (1) it was stated that when sera (human and ferret) which contained a high titer of influenza-neutralizing antibodies were added to virus suspensions, these virus suspensions would no longer agglutinate red cells, even though the immune serum was present in very low concentration. It was also shown that the amount of this inhibiting substance in a serum could be titrated by determining at what dilution of serum the inhibitory effect was no longer demonstrable. Before going further into the study of this phenomenon it was considered of importance to investigate the inhibitory properties of normal sera.

Serum inhibition tests were done on a number of normal ferret sera, using

both the PR8 and the Lee strain of virus. The sera were heated at 56°C for ½ hour, and serial twofold dilutions in saline were made To these dilutions a constant amount of infected allantoic fluid was added, and the amount of agglutination was read 1 hour after adding red cells The results with three such sera, shown in Table III, demonstrate that there was an inhibitory substance present in normal ferret serum, and that with low dilutions of serum the inhibition was active against both viruses tested Similar inhibition was demonstrated with mouse, rabbit, and guinea pig sera, but practically no inhibitory effect was demonstrable with horse serum This inhibitory factor was partly destroyed by heating the serum to 56°C for ½ hour The possible presence of a normal inhibitory substance in human serum will be discussed later

Although the presence of an agglutination inhibiting substance in normal serum complicated the titration of inhibition due to influenza virus antibodies, it was relatively easy to obviate this difficulty When a difference in inhibition titer between two sera from the same individual or from the same animal was measured, the change in titer was assumed to be due to antibodies, since the amount of normal inhibitory substance was probably the same in both sera Also the inhibition titer of most immune ferret sera was very high, considerably beyond the range where the normal inhibition was active When titers from different animals of the same species were compared, the immune serum dilutions were made in normal serum from the same species so that the total amount of serum in each tube (normal plus immune) was the same. This made it possible to dilute out the inhibitory effect due to antibodies while the normal inhibitory effect was kept constant.

The Serological Specificity of the Serum Inhibition Test Using Immune Ferret Sera.—The antigenic relationships of various strains of influenza virus have been extensively studied by several authors These studies have shown that influenza A virus strains are antigenically distinct from the Lee and T.M strains of influenza B virus (3, 4) Also differences have been demonstrated between various strains of influenza A virus (5–7), and swine influenza virus has been shown to be distantly related to influenza A strains (8, 9) The following experiment was performed to see whether or not the inhibition test is sufficiently sensitive to detect the antigenic differences and similarities demonstrable by other methods.

The PR8 and W.S strains of influenza A virus the Lee strain of influenza B virus and swine influenza virus were used. Allantoic fluid preparations of the virus strains and ferret antisera were prepared as described under Methods Since high concentrations of normal ferret serum inhibit agglutination of the red cells, all immune serum dilutions were started at 1 32 All dilutions of the immune sera were made in normal ferret serum so that the final concentration of serum in each tube (normal plus immune) was 1 32 Agglutination titrations of the four virus preparations were also

TABLE III

Agglutination Inhibition Titrations on Normal Ferret Sera Using PR8 and Lee Viruses

Serum No	PR8 (A)								Lee (B)							
	Dilution of serum						Virus control	Titer	Dilution of serum						Virus control	Titer
	1 4	1 8	1 16	1 32	1 64	1 128			1 4	1 8	1 16	1 32	1 64	1 128		
A 17-67	0	++	++++	++++	++++	++++	++++	8	±	+	+++	++++	++++	++++	++++	12
A 17 52	0	+	++	++++	++++	++++	++++	16	0	±	++	++++	++++	++++	++++	16
A 17 72	0	0	0	++	+++	++++	++++	32	0	±	+	+++	++++	++++	++++	24

TABLE IV

Serum Inhibition Tests Using Four Virus Strains and Their Respective Ferret Antisera

Serum	Virus strain	Dilution of serum										Virus control	*In vitro* titer	Neutralization titer in mice
		1 32	1 64	1 128	1 256	1 512	1 1 024	1 2 048	1 4 096	1 8,192	1 16 000			
PR8	PR8	0	0	0	0	0	0	0	+	+++	++++	++++	6,000	16,000
	W.S	0	0	0	0	0	++	+++	+++	+++	+++	+++	1 024	1,024
	Swine	±	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	96	32
	Lee	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
W S	PR8	0	0	0	0	+	++++	++++	++++	++++	++++	++++	768	256
	W.S	0	0	0	0	0	0	+	+++	+++	+++	+++	3 000	2,048
	Swine	0	0	±	++	+++	+++	+++	+++	+++	+++	+++	256	12
	Lee	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
Swine	PR8	+	++	++++	++++	++++	++++	++++	++++	++++	++++	++++	64	4
	W S	0	0	0	0	+	+++	+++	+++	+++	+++	+++	768	16
	Swine	0	0	0	0	0	0	0	++	+++	+++	+++	4,000	1 024
	Lee	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
Lee	PR8	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	<32	<4
	W S	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
	Swine	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	<32	<4
	Lee	0	0	0	0	0	0	0	++	+++	+++	+++	4,000	8 000

made in 1 32 normal ferret serum The dilution of allantoic fluid used in the inhibition tests was twice the concentration which caused a two plus reaction in the agglutination titrations. With the PR8 Lee, and swine strains a final concentration of allantoic fluid of 1 32 was used, and for the W S strain a concentration of 1 12 The inhibition titrations were set up as described under Methods, adding each virus to dilutions of each of the four sera The results are recorded in Table IV

In examining the antigenic relationships shown by this inhibition test it should be noted that each serum inhibited the agglutination by its homologous virus to approximately the same dilution (3000 to 6000) The agglutination of red cells by the Lee virus suspensions was not inhibited by any of the heterologous sera, nor did the Lee antiserum significantly inhibit the reaction due to any of the heterologous viruses The PR8, the W.S, and the swine influenza viruses all showed some interrelationship by this method, and of these the PR8 and the W.S showed the greatest similarity Both the W S and the PR8 sera gave the higher titer with the homologous virus and a four to six times lower titer with the heterologous virus. Swine virus appeared more closely related to the W.S strain than to the PR8 strain, both when swine antiserum was tested against PR8 and W.S virus, and when PR8 and W.S antisera were tested against swine virus

Neutralization tests in mice were done with these same sera and virus preparations in order to compare the *in vitro* results with one of the generally accepted procedures for the demonstration of antigenic relationships Approximately 30 fifty per cent mortality doses of each virus were used for the test. The neutralization titers obtained are shown in the last column of Table IV An interpretation of the *in vivo* results yields essentially the same conclusions about the antigenic relationships of these strains as were obtained from the inhibition test. The only qualitative exception was that the PR8 serum had a higher titer than the W S serum when tested with swine virus in mice, while the reverse was true with these sera against swine virus in the *in vitro* test.

Agglutination Inhibition Titrations with Human Sera—Although we have previously reported the demonstration of a rise in agglutination inhibition titer in serum taken from a patient during convalescence from influenza A, it was considered necessary to ascertain how constant this finding would be in a large series of cases.

Forty-four pairs of acute and convalescent sera from cases of influenza A were used. Half the sera were from an institutional epidemic occurring during the winter of 1938–39 (10), and the remainder were from institutional epidemics in Alabama in the winter of 1940–41 (11) The convalescent serum from each patient had a mouse neutralization titer at least four times as high as that of the acute phase serum, when tested with PR8 virus. For the *in vitro* test twofold dilutions of serum were made in saline, beginning with a final serum dilution of 1 8. A final dilution of 1 64 allantoic

fluid, containing PR8 virus, was used. This concentration of allantoic fluid was four times the amount which causes two plus agglutination in an agglutination titration. In every case the titer of the convalescent serum was at least two, and usually four or more, times higher than the titer of the acute serum.

Similar tests were run on sera from individuals who had been vaccinated with a complex influenza vaccine which contained formalinized PR8 virus (12). Sera were taken before and 2 weeks after vaccination. Fifty-five pairs of sera were tested. Each serum was titered by means of the mouse neutralization test, and in every case the postvaccination serum showed at least a twofold rise in neutralizing antibodies against PR8 virus. When these sera were tested *in vitro*, a corresponding rise in inhibition titer following vaccination was demonstrated in every case.

Pairs of serum from fourteen normal individuals obtained at a 1-year interval were included in this experiment. None of the subjects gave any history suggestive of

TABLE V

Serum Inhibition Titrations on an Immune Ferret Serum Using Different Quantities of Virus

Dilution of allantoic fluid	Dilution of serum*									Virus control
	1:256	1:512	1:1 024	1:2 048	1:4 096	1:8 192	1:16 000	1:32 000	1:64 000	
1:4	0	±	++	++++	++++	++++	++++	++++	++++	++++
1:8	0	0	±	+++	++++	++++	++++	++++	++++	++++
1:16	0	0	0	±	+++	++++	++++	++++	++++	++++
1:32	0	0	0	0	±	++	++++	++++	++++	++++
1:64	0	0	0	0	0	±	++	+++	++++	++++
1:128	0	0	0	0	0	0	±	++	+++	+++
1:256	0	0	0	0	0	0	0	±	±	++
1:512	0	0	0	0	0	0	0	0	0	0
1:1 024	0	0	0	0	0	0	0	0	0	0

* All serum dilutions were made in normal horse serum plus saline, so that the final concentration of serum (normal plus immune) was 1:8 in every tube.

influenza during the intervening period, and none of the pairs of sera showed any difference in the virus neutralization titer. There was likewise no demonstrable difference between the inhibition titers of each of the pairs when tested by the *in vitro* technique.

The foregoing experiments, both with ferret and with human sera, demonstrate a consistent qualitative parallelism between neutralization titer in mice and agglutination inhibition titer *in vitro*. Before attempting to demonstrate a more quantitative correlation between the results of the two tests it was considered necessary to investigate the variables entering into the *in vitro* titration in order to find out what precautions are essential to obtain reproducible results.

Factors Affecting the Results of the Inhibition Test.—In the following series of experiments inhibition tests were performed with a PR8 ferret serum and its homologous virus. In each experiment one variable was introduced into the titrations, in order to see what the effect on the inhibition end point of the serum would be.

In the experiment shown in Table V the ferret immune serum was titrated with a number of different concentrations of the homologous virus With only minor variations the serum inhibition end points obtained with different quantities of virus were in simple inverse proportion to the amount of virus used in the test This simple relationship between the amount of virus used and the end point obtained makes it very convenient and easy to compare inhibition tests where different amounts of virus were used The theoretical implications of this experiment will be more fully considered later

In another experiment a number of titrations were done on the same serum using different concentrations of chicken red cells for the tests, the results of which are shown in Table VI While it was difficult to make comparable readings in tests in which there was such a wide variation in red cell concentration, nevertheless it was obvious that the end point decreased as the concentration

TABLE VI

Serum Inhibition Titrations on an Immune Ferret Serum Using Different Quantities of Cells

Final cell concentration	Dilution of serum							Virus control	Red cell control
	1 256	1 512	1 1,024	1 2,048	1 4,096	1 8,192	1 16,000		
per cent									
4 0*	0	0	0	0	±	+++	++++	++++	0
2 0	0	0	0	0	+	+++	++++	++++	0
1 0	0	0	0	±	+	++++	++++	++++	0
0 5	0	0	0	+	+	++++	++++	++++	0
0 25	0	0	+	++	++	++++	++++	++++	0
0 12	0	±	±	++	++	++	++	++	0

* There were 360,000 red cells per c. mm. in the 4 per cent concentration.

of cells used decreased In this test it was found that by far the most satisfactory concentration of cells for ease in reading the tests was the final 1 per cent concentration used throughout the other experiments in this paper

Duplicate serum inhibition tests were run simultaneously at 37°C and at 27°C with no obvious difference in end point. Although a wider variation in temperatures produced definite changes in the end points, the ordinary changes in room temperature were not sufficient to alter the results significantly

Duplicate titrations were done with fifteen human sera (high and low titer) and one ferret serum, using six different lots of red cells. Each lot of red cells was prepared separately, and the various lots were stored at 4°C for from 1 to 6 days before use There was no systematic tendency of any lot of cells to give high or low results with the various sera. The length of storage apparently had no effect on their agglutinability In general, duplicate titrations of a given serum gave the same result. In 25 per cent of the tests there was a variation in end point of one-half dilution, and very occasionally there was a variation of a full dilution From these data it was evident that the use of

different lots of cells was not a serious source of error in the test if the cells had been prepared in a uniform manner

One factor of considerable importance in obtaining uniform results with the test is the length of time between the addition of red cells and the final shaking for thorough mixing When the tubes of a serum titration were shaken immediately following the addition of cells, the titer obtained was definitely lower than that of a duplicate titration, when the tubes were shaken 5 minutes after adding the cells In some of the experiments reported here the cells were added with an automatic pipetting machine, which delivered the suspension with such force that sufficient mixing took place at once

Of some importance also was the question of the length of time necessary for the virus-antibody reaction to take place after serum and allantoic fluid had been mixed together A number of titrations of an immune ferret serum were done In each case the red cells were added after the virus and serum had stood mixed together for different intervals Whether the cells were added immediately after adding virus to the dilutions, or whether the virus-serum mixture was allowed to stand for ½ hour before adding the cells, the end point was the same This rapid interaction between virus and serum obviates any necessity of incubating these two reagents together for any fixed period in performing the inhibition tests

The preceding experiments emphasize the fact that the serum inhibition test can be performed in a fairly simple manner, without elaborate equipment or precautions, and that under these conditions quite reproducible results may be obtained

Quantitative Correlation between Virus Neutralization and Agglutination Inhibition Titers of Serum—After it had been shown that the *in vitro* test gave results which were qualitatively similar to those obtained with the mouse neutralization test and that the results were reproducible, the next step was to see how the serum inhibition titer was related quantitatively to the virus neutralization titer on widely different sera

The sera used were from sixteen persons acutely ill with influenza A, from twenty-one convalescent from influenza A, from twenty-two normal individuals, and from thirty-one who had been vaccinated 2 weeks previously with a complex chick embryo influenza vaccine Mouse neutralization tests were done on all these sera, using 200 lethal doses of PR8 mouse passage virus, and the sera were diluted in twofold steps The *in vitro* tests were done with a 1 64 dilution of allantoic fluid containing PR8 virus The two plus end point of this same fluid in an agglutination titration was 1 256 The end points obtained in the inhibition tests were plotted against the neutralizing capacity as calculated from the mouse neutralization end points (2)

The results are recorded in Fig 1, in which it can be seen that the points tend to fall along a straight band On the whole, the correlation is fairly good between the two methods of measurement over a wide range of antibody levels

and there are no widely discrepant points The width of the band can be accounted for by the errors in the tests, especially the mouse neutralization test.

This correlation is more significant when one considers that the sera were from four groups of individuals who had had widely differing experience with influenza virus, and yet there is no systematic deviation or scattering of any

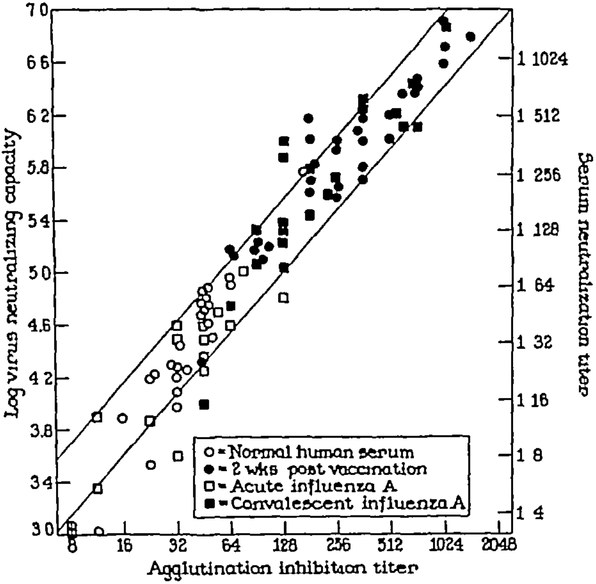

FIG 1 Correlation of serum inhibition titer with virus-neutralizing capacity of various human sera.

of these groups The evidence contained in this figure is the best we have obtained so far that the *in vitro* test measures either neutralizing antibodies or something which fairly consistently parallels neutralizing antibodies.

DISCUSSION

The advantages of the agglutination tests over mouse tests for influenza virus and influenza antibody titrations are sufficiently clear so that detailed discussion seems unnecessary While the agglutination titer of a fresh virus suspension shows a fair correlation with the mouse infective titer in the tests we have

recorded, much more work will be necessary to show what the agglutination titer actually measures. In any case, it is a measurement of a new quality of virus suspensions and as such merits further study. The serum inhibition titrations clearly measure influenza antibodies, and from the quantitative results obtained it seems that the test may very likely measure neutralizing antibodies. The only other *in vitro* test described for measuring influenza antibodies is the complement fixation test. While no direct comparisons have been made between the results of this test and the inhibition test, the outstanding advantage of the latter is its ease of performance and standardization.

If these agglutination tests should come into general use, they would be of value in comparing results from different laboratories, provided some sort of standard procedure were used. Based on our present experience, the procedures outlined under Methods seem to be a satisfactory starting point. For serum titrations it would be necessary to state only the number of agglutination units used for the test. We have generally used four times the amount of virus necessary to cause two plus agglutination. All sera should be heated to 56°C for 30 minutes before use. This was not done with the human sera in the experiments reported here, but it has been found since these tests were performed that such heating lowers the inhibition end point of low titer human serum but does not affect the end point of high titer serum. Heating probably inactivates a "normal" inhibitory substance present in such sera.

The fact that eggs inoculated with a number of different influenza virus strains yield allantoic fluids of approximately the same agglutination titer is of some theoretical interest. A number of other strains, besides those employed in the present work, have been cultivated in eggs, and all strains so far tested have shown this same constancy in titration end point by agglutination. This similarity in end point suggests two things: (1) that different strains of influenza virus, when grown in eggs, reach approximately the same virus particle concentration in allantoic fluid, and (2) that the individual virus particles of different strains of virus have the same capacity, or nearly so, to agglutinate red cells. If the second assumption is correct, then the agglutination titration method is a simple way of determining the relative number of particles in suspensions of different strains, independently of pathogenicity, something mouse titration fails to tell. In any case, the test provides a method for studying immunologically and in other ways strains which have not been adapted to mice.

The results of the serum inhibition titrations, using different amounts of virus, show that the same amount of serum inhibits agglutination by a constant amount of virus, regardless of the volume in which the reaction takes place, at least over a considerable range of dilution. This result is in keeping with the classical work on antigen-antibody reactions of Dean and Webb (13), which

showed that the optimal proportions point for antigen antibody reactions was the same, regardless of the volume in which the reaction was carried out. This would tend to show that influenza virus-antibody reactions are the same as other antigen antibody reactions, at least in this respect.

This result, however, is in apparent conflict with those of Horsfall (2) on the shift in serum neutralization end point in mice, where different amounts of virus are used in the test. Horsfall found that if it took x cc. of serum to neutralize a certain quantity of virus, it took $x/5$ cc. to neutralize one-tenth as much virus. In the mouse test the serum becomes less efficient on dilution. Since the reasons for the discrepancy are not clear, it will suffice to point out that the mouse test is a very complicated test and involves the interplay of many forces over a period of about 10 days, while the *in vitro* test is relatively simple. Because of the complexity of the mouse test, it seems probable that the agglutination inhibition test gives a more accurate picture of the *in vitro* combining ratios of virus and antibody.

SUMMARY

1. The agglutination titer for chicken red cells of freshly prepared or carefully stored suspensions of PR8 influenza virus, that is to say virus of maximum pathogenicity, was found to be proportional to the mouse lethal titer of the same preparations.

2. The agglutination titer of infected allantoic fluid procured in a standard way is relatively constant, regardless of the influenza strain used and its pathogenicity for mice.

3. Virus preparations inactivated by heat or storage may retain their agglutinating power.

4. Certain animal sera contain a partially heat labile factor which, in low dilution, inhibits the agglutination of chicken red cells by influenza A and influenza B viruses.

5. The agglutination inhibition test, using ferret and human sera, gives qualitative data regarding influenza antibodies which are similar to the information obtained on the same sera by means of the virus neutralization test.

6. There is a definite relationship between the agglutination inhibition titer and the virus neutralization titer of a serum. On a logarithmic scale of both variables, this relationship is essentially linear within the range investigated.

7. The agglutination inhibition titer of immune ferret serum is inversely proportional to the amount of virus used in the test.

Since this paper went to press, McClelland and Hare (14) have published results confirming the work in our earlier publication (1) on the adsorption of influenza virus on red cells and the use of agglutination for measuring influenza antibodies.

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DEMONSTRATION OF AGGLUTININS FOR BARTONELLA BACILLIFORMIS

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Human bartonellosis, known as Carrion's disease, manifests itself in two distinct phases. The first phase, Oroya fever, is a severe hemolytic anemia, the result of the invasion of the blood stream by the organism, *Bartonella bacilliformis*. Blood cultures are strongly positive at this stage of the disease, and the red blood cells as well as the cells of the reticulo-endothelial system, are heavily parasitized by *Bartonella*. The second phase, verruga peruana, is characterized by the appearance of the typical cutaneous eruption from the nodules of which *B. bacilliformis* may be recovered. Bartonellosis is endemic in certain regions of Peru and has more recently been found to be so in Colombia and Ecuador. In these endemic regions, the second stage (verruga) is more commonly encountered. Less frequently, the anemic stage, when very severe ends fatally before the development of the eruption. A large percentage of the native population of the endemic zones in Peru have had some form of bartonellosis, mild or severe, and are able to live there without further clinical evidence of infection. Second attacks of acute fever without the eruption are apparently unusual but second attacks of verruga with minimal febrile illness are not rare. From these facts, it is evident that in most of those individuals who have contracted bartonellosis at least once a definite and long lasting immunity develops against the infection. This immunity on the part of the natives is in strong contrast to the often fatal infection which develops in individuals from non-endemic zones who spend but a few nights in the endemic zones without adequate protection against the night flying sandfly vector. Somewhere in between these two extremes of complete immunity on the one hand and complete susceptibility on the other if one

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speculates in terms of immunology, must lie the so called carriers, thought by Weinman and Pinkerton (1) to represent a possible reservoir of infection These authors have estimated the number of such individuals in one locality of the endemic region of Peru at about 5 to 10 per cent of the total population They show no outward sign of infection, but *B bacilliformis* may be recovered from their blood, which may or may not show a slight anemia It is evident from the foregoing rather empirical facts that the fundamental immunological pattern of human bartonellosis remains still to be worked out The present report is a step toward the solution of this complex problem

The work which follows deals with the production and the demonstration of specific agglutinins for *B bacilliformis* in rabbits, and their demonstration in the sera of human beings with bartonellosis In the literature on Carrion's disease, the possibility of agglutinating *B bacilliformis* with specific sera of one sort or another has been considered from time to time by various authors (2–7) In no instance is there any indication of clear-cut experiments on this aspect of the problem, and in all cases the only tangible conclusion that has been reached is that any demonstration of agglutinins is handicapped by the difficulties encountered in preparing suspensions of the organism suitable for use as an antigen

Materials and Methods

Media and Cultivation of Bartonella —Heretofore, the only media that have yielded fairly satisfactory growth have been ordinary blood agar and the semisolid medium of Noguchi and Battistini (3) originally designed for the cultivation of *Leptospira* The former medium yields colonies of tightly packed organisms on the slant, with many of the colonies embedded in agar, the latter medium a variable and diffuse growth, with aggregates of smaller satellite granules, in the upper 1 to 2 cm of the medium Both types of medium present obstacles to the preparation of a homogeneous suspension, chief among which is that resulting from the presence of agar, from which the organisms are inseparable Two types of medium recently developed by Geiman in Boston (8) have effectively removed these obstacles

The first is a solid medium Special base agar is made with 2 per cent shredded agar, 2 per cent tryptone or proteose peptone, 0 5 per cent sodium chloride, in distilled water The medium, as finally used, is composed of 75 per cent base agar, adjusted to pH 7 6–7 8 and cooled to 45°C , and 25 per cent fresh rabbit or sheep serum, or defibrinated whole blood To the whole medium, a small amount (0 2 per cent of the total volume of medium) of an ascorbic acid glutathione solution is added This medium gives a profuse growth, which follows the streak of the loop on the slant and becomes grossly visible in 24 to 48 hours, reaching a maximum in 10 to 14 days The optimal temperature for incubation is 28°C. The growth may be easily scraped from the surface, and collected from the water of condensation at the bottom of the tube

6 2 2 16

The second is a liquid medium It is composed of three parts of a 1 per cent solution of tryptone adjusted to pH 7 6–7 8 to one part fresh rabbit serum and to the whole medium is added a small amount (0 2 per cent of the total volume of medium) of the ascorbic acid glutathione solution Growth becomes visible as a finely divided sediment within 24 to 48 hours and reaches a peak on the 10th day when incubated at 28°C It has been found by Geiman that maximum growth takes place at the bottom of the container when the depth of this medium is from 0 5 to 0 75 cm Small (50 cc.) Erlenmeyer flasks are used, to afford as large an area as possible at this optimal depth for the growth which takes place on the bottom of the flask The present writer has found that ordinary test tubes (14 cm × 2 cm.), bent to resemble a hockey stick in shape with the short arm at the open end of the tube, serve better than Erlenmeyer flasks for the cultivation of *Bartonella* The tubes are stored horizontally the open end directed upward to prevent outflow of the medium with which the tubes are filled to half of their horizontal depth, *i e* 0 5 to 0 75 cm Growth takes place along the bottom of the converted test tube and the yield is about equal to that obtained with the flasks There is further the added advantage of convenience and less danger of contamination than that encountered with the flasks.

Preparation of Antigen—The organisms harvested from both types of medium when washed two or three times in normal saline and resuspended in saline buffered to pH 8 2–8 4, constitute a homogeneous antigen admirably suited for agglutination tests It has been found more recently that clumps of organisms harvested in a similar manner but stored as a coarse suspension in normal unbuffered saline can after about a week of such storage be easily emulsified by churning with a capillary pipette (9) The coarser particles in either type of suspension are allowed to settle or are thrown down by slow centrifugation In both cases dark field examination reveals a fairly regular distribution of single organisms with occasional clumps of never more than 5 to 10 (Fig 1) These occasional clumps are just visible with a 10 or 14 power hand lens

Technique of Agglutination Test—The technique of the test for agglutinins has been designed to allow rigid economy of materials and at the same time permit accurate gradation of titre Small fermentation tubes (7 5 cm. × 0 75 cm) are used and the total final volume confined to about 0 1 cc. The tubes after proper serial dilution of the sera and the addition of the antigen are incubated for 4 to 6 hours at 40°C and are then read with the aid of a 10–14 × hand lens and after 24 hours on ice they are again read In the strongly positive tubes, there is a heavy and tightly coherent precipitate with clear supernatant liquid. Dark field examination (Fig 2) reveals a definite heavy clumping of the single organisms and the small groups of organisms seen in the control (Fig 1) In the negative and the control tubes when examined with the hand lens there is never more than a very slight sediment, which is easily dispersed

Immunization of Rabbits—To obtain specific agglutinins a series of rabbits was immunized by repeated injections of living *B bacilliformis* into the marginal vein of the ear The organisms from a tube of liquid culture after 10 days to 2 weeks incubation or a week s growth from a solid slant washed twice with saline, was given every 5th day over a period of 60 days each animal having received 12 to 13 inocula

FIG 1 Suspension of *Bartonella bacilliformis* in normal saline Dark field photomicrograph × about 4000, oil immersion

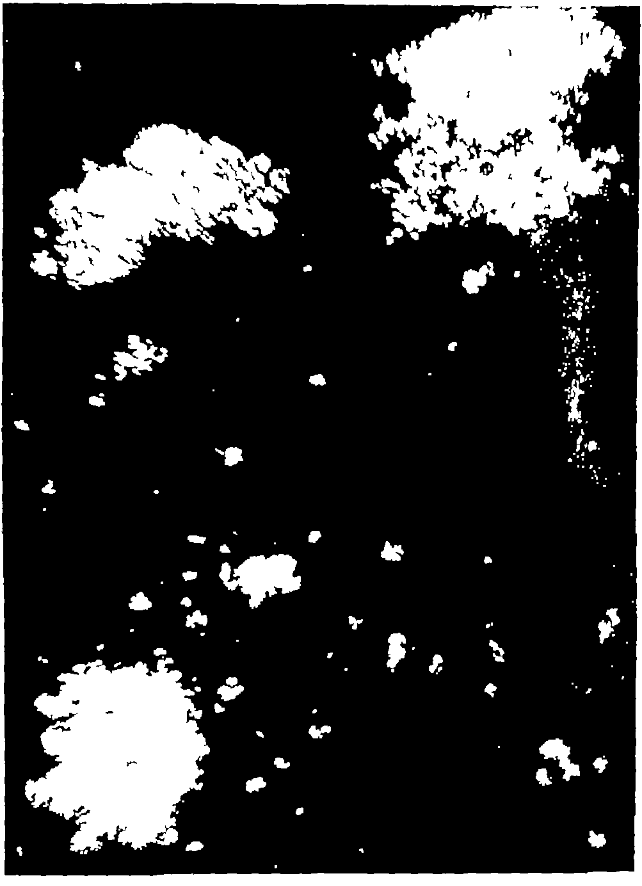

FIG 2 Agglutination of *Bartonella bacilliformis* by serum of immunized rabbit 1 in dilution of 1 640 Dark field photomicrograph × about 4000 oil immersion

tions in all at the end of that time Two rabbits were treated in a manner different from the others One of these (rabbit 1) was inoculated every 3rd or 4th day with the material and by the method described above, 15 inoculations having been given at the end of 50 days The other rabbit (rabbit 2) had been splenectomized, and into the anterior chamber of both eyes, chopped rabbit embryo had been successfully implanted *B bacilliformis* was then injected into both anterior chambers, and given in large quantity intratracheally, in an attempt to produce some tangible evidence of pathogenic effect 2 weeks after the intratracheal and intraocular inoculations, one eye, which was then aseptically removed, showed a marked pannus formation, with ingrowth of vessels into the cornea, and the other eye showed nothing Cultures taken from the implanted tissue and the aqueous humor of the enucleated eye showed a heavy growth of *B bacilliformis*, but blood cultures were negative Serum for agglutination was taken at this time At no time did any of the animals, no matter in what way they had been inoculated, show any signs of systemic reaction which could have been interpreted as having resulted from the inoculation

Results with Rabbit Sera

The results obtained in testing the sera of these rabbits for agglutinins by the method described are shown in Table I In the case of rabbits 1 and 3, in order to determine the rate at which antibodies might be appearing, serum was taken after one-half the total projected number of inoculations had been given, and showed a moderate titre of agglutinins The titre as determined after all inoculations had been given was proportionately higher in all cases The definitely higher titre of rabbit 1 as compared with that of rabbits 3, 4, and 5 might be explained by the larger number of inoculations given at shorter intervals to rabbit 1 (15 inoculations in 50 days as opposed to 12 in 60 days), although this difference in titre may only be an indication of wide variation in the strength of response among individual animals There was in the case of rabbits 1, 3, and 4 a marked falling-off of titre at the end of 1 month after the last inoculation Rabbit 5 showed a somewhat higher titre of agglutinins than did the other two, and maintained this higher titre longer than the latter, although there was a slight but definite falling-off at the end of 1 month after the last inoculation In the case of rabbit 2 the titre of agglutinins obtained as a result of the simultaneous intraocular and intratracheal inoculations described above, lies midway between that of rabbit 1 and those of rabbits 3 and 4 There is no indication, in this single experiment (rabbit 2), as to which method of inoculation may have resulted in so high a titre, or whether both were necessary to produce it The fact that cultures made from the eye fluid and the infected implanted embryonic tissue were strongly positive after 2 weeks suggests the possibility that these two foci were a source of continuous stimulation to the production of antibodies by the host

Results with Human Sera

Table II summarizes the findings with a small group of human sera Two cases in which the anemic manifestation had been minimal and the verrucous

TABLE I

Agglutination of Bartonella bacilliformis by Serum of Immunized Rabbits

Rabbit	Time	Final dilution of serum						
		1:10	1:20	1:40	1:80	1:160	1:320	1:640
1	Before immunization	–	–	–	–	–	–	–
	After 7 intravenous inoculations in 22 days	4+	4+	4+	4+	2+	–	–
	5 days after last of 15 intravenous inoculations given over 50 days	4+	4+	4+	4+	4+	4+	3+
	28 days after last intravenous inoculation	4+	4+	4+	3+	2+	–	–
2	Before intratracheal and intraocular inoculation	–	–	–	–	–	–	–
	13 days after intratracheal and intraocular inoculation	4+	4+	3+	3+	+	–	–
	34 days after intratracheal and intraocular inoculation	4+	4+	4+	4+	3+	3+	–
3	Before immunization	–	–	–	–	–	–	
	After 7 intravenous inoculations in 33 days	2+	2+	2+	+	+	–	
	3 days after last of 13 intravenous inoculations given over 60 days	4+	4+	4+	3+	2+	–	
	36 days after last intravenous inoculation	4+	4+	4+	+	–	–	
4	Before immunization	–	–	–	–	–	–	
	5 days after last of 12 intravenous inoculations given over 60 days	4+	4+	3+	2+	2+	+	
	37 days after last intravenous inoculation	3+	3+	3+	+	–	–	
5	Before immunization	–	–	–	–	–	–	
	3 days after last of 13 intravenous inoculations given over 64 days	3+	3+	3+	3+	2+	2+	
	36 days after last intravenous inoculation	3+	3+	3+	2+	2+	2+	

4+, *heavy coarse precipitate at bottom of tube clear supernatant liquid.*

3+ to + gradually decreasing amounts of precipitate but obvious agglutination of suspended *organisms* when compared with saline and normal serum controls.

– no agglutination

stage more extensive (cases 1 and 5) showed the highest titre of agglutinins, and the two severe cases of Oroya fever (cases 2 and 3) the lowest. Thirteen normal human sera were consistently and entirely negative Although definite

conclusions cannot be drawn from so small a series of cases, the possibility that the appearance of the eruption is associated with the formation of a demonstrable titre of circulating antibodies may be considered The development of the eruption is invariably regarded by experienced clinicians as a favorable prognostic sign The fact that the serum of the so called "immune" (case 6) contained a minimal titre of agglutinins may be analogous to the falling-off of titre noted with the rabbit sera A satisfactory test for the virulence of the organism concerned (and hence a susceptible laboratory animal) would, of course, be necessary in the final elucidation of the fundamental immunological process involved in human bartonellosis

TABLE II

Agglutination of Bartonella bacilliformis with Sera of Patients Suffering or Recovered from Bartonellosis (Carrion's Disease)

Case	Final dilution of serum			
	1 10	1 20	1 40	1 80
1	2+	+	+	±
2	+	+	−	−
3	+	±	−	−
4	+	−	−	−
5	2+	+	+	±
6	+	±	−	−
Normal human serum*	−	−	−	−

* Thirteen normal human sera were tested.

4+, heavy coarse precipitate at bottom of tube, clear supernatant liquid

3+ to +, decreasing amounts of precipitate at bottom of tube, but obvious agglutination of suspended organisms when compared with saline and normal serum controls

±, slight but definite agglutination, with no heavy precipitate

−, no precipitate or agglutination

The question of the relationship of *Bartonella* to the organisms of the *Rickettsia* group has also been explored With this possible connection in view, the six human sera tested for agglutinins for *Bartonella* were also tested with three strains of *Proteus*, after the manner of the Weil-Felix reaction The results are shown in Table III The two sera which agglutinated *Bartonella* most strongly also show the highest titres with two of the *Proteus* strains (sera of cases 1 and 5) Although a positive agglutination at a dilution higher than 1 50 is usually considered significant with the Weil-Felix reaction, there are occasional cases without a positive history of typhus whose sera are positive in much higher dilutions Since, also, the histories of the cases involved herein had not been probed for the possibility of typhus,

no definite conclusions can be drawn as to the meaning of the results obtained with these particular sera The question becomes even more obscure from lack of knowledge of the distribution of endemic typhus fever in Peru The only available figure is that of the total number of cases during the year

TABLE III

Agglutination of Proteus OX19, OXK, and OX2

Case	Strain	Final dilution of serum					
		1:8	1:16	1:32	1 64	1 128	1:256
1	Proteus OX19	4+	4+	4+	3+	2+	+
	Proteus OXK	3+	2+	2+	–	–	–
	Proteus OX2	4+	4+	2+	+	–	–
2	Proteus OX19	3+	2+	2+	+	–	–
	Proteus OXK	2+	–	–	–	–	–
	Proteus OX2	3+	2+	+	+	–	–
3	Proteus OX19	4+	3+	2+	+	–	–
	Proteus OXK	3+	2+	+	–	–	–
	Proteus OX2	4+	3+	+	–	–	–
4	Proteus OX19	2+	+	–	–	–	–
	Proteus OXK	4+	3+	+	–	–	–
	Proteus OX2	2+	–	–	–	–	–
5	Proteus OX19	4+	4+	3+	3+	+	–
	Proteus OXK	4+	4+	2+	+	–	–
	Proteus OX2	4+	4+	3+	2+	+	–
6	Proteus OX19	–	–	–	–	–	–
	Proteus OXK	3+	2+	2+	–	–	–
	Proteus OX2	+	–	–	–	–	–
Normal human serum	Proteus OX19	3+	+	–	–	–	–
	Proteus OXK	3+	2+	–	–	–	–
	Proteus OX2	3+	2+	+	–	–	–

All saline controls negative. *Bartonella* immune rabbit serum negative with all strains of *Proteus*

Tubes incubated 4 hours at 40°C.

Legend the same as that for Tables I and II.

1940, January through September, amounting to 667, no geographical allocation being given in the report (10) It is of importance to note, from an immunological point of view, that there was no agglutination of *Proteus* in significant titre by *Bartonella* immune rabbit serum

Origin of the Human Sera Represented in Tables II and III, with a Brief Description of Each Case

Case 1—Serum was taken during the 3rd month of a third mild attack of bartonellosis, at which time there was slight anemia, and extensive eruption Blood cultures were negative, having been positive during the 1st month of the recurring disease, during which time there had been no eruption

Case 2—Serum was taken on the 12th day of the disease There was severe anemia (red blood cell count 1 7 million), blood cultures were positive, and *Bartonella* were found in the blood film Death occurred on the 15th day of the disease

Case 3—Serum was taken on the 16th day of the disease There was severe anemia (red blood cell count 1 0 million), blood cultures were positive, and *Bartonella* were found in the blood film There was subsequent recovery, no *Bartonella* having been found in the blood films after the 26th day, at which time blood cultures also became negative

Case 4—Serum was taken during the 2nd month of a mild attack of verruga peruana, the eruption having appeared after 1 month of symptoms Laboratory data were not available, and the history not precisely known The eruption, however, was definitely that of verruga, but was confined to one large "mulaire" type of lesion on the left shin

Case 5—Serum was taken during the 2nd month of an attack of verruga peruana The appearance of the rash was preceded by symptoms of malaise and joint pains Laboratory data were not available, and the history not precisely known The eruption, however, was definitely that of verruga, and was far more extensive than that of case 4

Case 6—This was a so called "immune," who, having had verruga in childhood, has remained free from signs and symptoms of bartonellosis all of his life (30-odd years) in spite of frequent and prolonged sojourns in the areas where Oroya fever and verruga peruana are endemic.

DISCUSSION

The fact that it is possible to produce circulating antibodies in rabbits, and that circulating antibodies may be demonstrated in human beings with bartonellosis makes it certain that at some stage during the course of an infection with *B bacilliformis* there is a measurable immunological response on the part of the host This response, now proven by the formation of agglutinins, can be used as a basis for further investigation with three principal objectives 1) A clarification of the fundamental immunological sequence in human bartonellosis, 2) an evaluation of the properties of immune sera in the therapy of Oroya fever and of the effectiveness of vaccines in the prophylaxis of non-immune individuals, and 3) an elucidation of the antigenic and immunological characteristics of *B bacilliformis* With regard to the latter problem, preliminary studies have already been undertaken to determine possible immunological variations among *Bartonella* isolated from different sources and localities These experiments have indicated that no immunological differ-

ences between such strains can be demonstrated by the agglutination test, cross-agglutination between four Peruvian and two Colombian strains by homologous and heterologous sera to almost equal titre having been accomplished

SUMMARY

1 Methods of preparing a satisfactory antigen having been developed, a technique for performing an agglutination test with *B bacilliformis* is made available

2 As a result of repeated intravenous injection of living cultures of *B bacilliformis* at short intervals, rabbits have been found to produce a high titre of specific agglutinins which, under the conditions obtaining in the present series of experiments, begins to decline after about one month following the last inoculation

3 Sera from six cases of bartonellosis in different stages of its several manifestations have been shown by the agglutination test to contain a low but definite titre of circulating antibody

4 Several of these same sera have been shown to contain as well a significantly high titre of agglutinins for three strains of *Proteus* No definite conclusions can be drawn from this phenomenon since the case histories had not been probed for the possibility of typhus fever, and since the relatively high titres obtained with a few of the present sera may very well fall within the extremes of normal serum titres.

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THE EFFECT OF SULFAPYRIDINE UPON THE DEVELOPMENT OF IMMUNITY TO PNEUMOCOCCUS IN RABBITS

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Although the antibacterial action of sulfonamide compounds upon certain pathogenic microorganisms is now generally recognized, the effect of these drugs upon the immunological response of the animal or human host has not been completely elucidated In view of the value of sulfonamides in the therapy of pneumonia and other diseases caused by pneumococci and because of the essential contribution of immunity to recovery from such infections, it seemed desirable to investigate further the action of sulfapyridine upon the immune processes

In response to the antigenic stimulus of pneumococcus the tissues of man and certain animals are capable of elaborating type specific antibodies which circulate in the blood and can be determined by the appropriate techniques In experimental animals it is also possible to recognize the occurrence of active immunity as indicated by increased resistance to homologous infection which may be present before and may persist after the period when circulating anti bodies are detectable In the process of spontaneous recovery from pneumococcal infection disposal of the invading microorganisms by the phagocytic cells cannot be accomplished without the development of a specific immune response.

The sulfonamide compounds exert a direct bacteriostatic effect upon various microorganisms For these drugs to be effective therapeutically, however, the participation of specific humoral and cellular reactions of the host are essential requirements The question of whether, in addition to their direct actions upon the bacteria, these compounds may not also exert an effect upon the defense mechanism of a host has been subjected to both experimental and clinical investigation It has been demonstrated in mice injected intraperitoneally with pneumococci that the sulfonamide drugs do not affect the mobilization or activity of the phagocytic cells (1, 4) Although no evidence has been produced to show that these drugs stimulate or accelerate the process of antibody formation, the assumption that they exert no effect on the immune mechanism has not been unreservedly accepted.

In 1937 Buttle (2) observed that mice after recovery from infection with Pneumococcus Type I following treatment with the benzylidene Schiff's Base of 4 4'

diaminodiphenylsulfone were immune to reinfection with organisms of the homologous type In the following year Whitby (3) confirmed this observation in mice treated with sulfapyridine McIntosh and Whitby (4) found that at the end of 1 week mice which had survived 10,000 lethal doses of Pneumococcus Type I after oral treatment with sulfapyridine were completely immune to reinfection with a million fatal doses of pneumococci of the homologous type This immunity was related quantitatively to the infective dose and was type specific An inoculum of 1 million heat-killed pneumococci stimulated in vaccinated mice a degree of immunity comparable to that induced in mice following recovery under drug therapy from an otherwise fatal infection with 10,000 living pneumococci In the latter instance the surviving animals on reinoculation were immune to an amount of virulent culture 100-fold greater than that used in the primary infection The active immunity induced by vaccination or resulting from infection was demonstrable within 3 days By the 4th or 5th day immunity was usually solid, and on the latter day the sera conferred passive protection on other mice In the sera of mice immunized with *Eberthella typhosa* agglutinins were demonstrable by the 3rd day and increased in titer between the 4th and 7th days irrespective of whether or not sulfanilamide had been administered These authors concluded that "the administration of sulphonamide drugs has no stimulating action on the body defenses, nor does such administration affect the quality, quantity, or speed of production of recognized specific antibodies" Experimental confirmation of Whitby's observation was reported in 1939 by Callerio (5) and MacLeod (6) MacLeod compared the survival rate following sulfapyridine therapy with the immune response in untreated animals, employing Pneumococcus Type I, a good antigen, and Pneumococcus Type III, a relatively poor antigen He found that in mice infected with 10^{-2} cc of a virulent culture of Pneumococcus Type I and treated with sulfapyridine, 95 to 100 per cent survived, whereas in mice similarly treated but infected with Type III pneumococci less than 10 per cent survived In two groups of untreated mice immunized respectively with comparable doses of heat-killed pneumococci of Types I and III and infected 5 days later with 1,000 fatal doses of a virulent culture of the corresponding type, 95 to 100 per cent of those in the Type I group were immune while none of the Type III mice survived An apparent correlation was observed not only between the survival rate and the relative antigenicity of the respective types of pneumococci, but also between the differences in the time required for the development of antibodies and the length of time during which it was necessary to continue sulfapyridine treatment Larson, Bieter, and Levine (7, 8) demonstrated that rabbits which recovered from intradermal infection with Pneumococcus Type II following treatment with sulfapyridine were highly resistant to reinfection with the homologous type of organism 30 or more days later

In the sera of patients with pneumococcal pneumonia the appearance of type specific antibodies at or about the time of spontaneous recovery is a well recognized phenomenon However, in patients treated with sulfonamide drugs the appearance of these antibodies does not necessarily coincide with the defervescence frequently observed early in the course of illness

Edwards, Kircher and Thompson (9) tested 26 pneumonia patients treated with sulfapyridine for the development of dermal reactions to homologous type specific capsular polysaccharide as well as for the presence in their sera of type specific agglutinins. They concluded from the results of their observations that the immune responses which occur naturally in the course of untreated pneumonia are not affected by drug therapy

Wood and Long (10) demonstrated specific protective antibodies in the sera of 10 out of 12 patients with pneumococcal pneumonia who were treated with sulfapyridine. They concluded that the antibody response in patients treated with sulfapyridine is similar to that observed in untreated patients.

Finland, Spring, and Lowell (11) studied the bactericidal power of whole blood as well as the development of homologous type specific agglutinins and mouse protective antibodies in the sera of a large number of patients with pneumococcal pneumonia treated with sulfapyridine These investigators reported that mouse protective antibodies and agglutinins were rarely demonstrable in patients blood before the 6th or 7th day although defervescence frequently occurred earlier They concluded that the antibody response in patients treated with sulfapyridine was comparable in all respects to that observed in spontaneous recovery

Fox, Rosi and Winters (12) determined the time of appearance of specific antibodies in the sera of 50 adult patients treated with sulfapyridine In all patients with proven pneumococcal pneumonia type specific agglutinins were demonstrable in the blood at some time in the course of illness These same authors reported subsequently (13) that in a larger series 90 per cent of the patients developed type specific agglutinins by the end of the 2nd week of illness and that 50 per cent of those tested gave true positive skin reactions to the homologous type specific polysaccharide.

Kneeland and Mullikin (14) tested the sera of 19 patients with pneumonia who were treated with sulfapyridine. They observed that positive precipitin reactions to the homologous capsular polysaccharide appeared in the sera of only four of their patients upon recovery In a subsequent paper these authors (15) reported that type specific precipitins were detectable in the sera of only 8 of 30 sulfapyridine-treated patients as compared with 16 of 21 patients treated with sulfathiazole. They suggested that sulfapyridine by its action upon the invading microorganism diminishes the stimulus to antibody formation whereas sulfathiazole, which they considered a less powerful bactericidal agent, exerts this effect to a lesser degree.

Bukantz and de Gara (16) demonstrated the presence of homologous type specific precipitins during recovery in the sera of nineteen of sixty patients with pneumonia who were treated with sulfapyridine.

In the animal experiments thus far reported, with the exception of those of McIntosh and Whitby, tests for resistance to homologous reinfection have been made at a time when immunity had reached or passed the peak of maximal response. In clinical studies the detection of circulating type specific antibody is necessarily limited by the methods which can be employed The free circulating antibodies detectable *in vitro* by serological techniques probably repre-

daily from the marginal ear veins Animals which died were examined at autopsy and cultures were made of the heart's blood

Determination of Circulating Antibodies Precipitins—Precipitin tests were carried out using 0 3 cc. respectively of undiluted rabbit serum and a 1 25,000 dilution of the acetylated pneumococcus Type I capsular polysaccharide After thorough mixing of the contents, the tubes were incubated at 37°C for 2 hours, refrigerated overnight, and read the following morning

Agglutinins—Tests for type specific agglutinins were carried out using serial dilutions of each serum Formalin-killed cells of Pneumococcus Type I, suspended in a volume of saline equal to that of the original culture were employed To 0 3 cc. of the serum dilutions, 0 3 cc. of antigen was added and after thorough mixing the tubes were incubated at 37°C, refrigerated overnight and read the following morning

Mouse Protective Antibodies—The sera of rabbits before immunization and at intervals of 48, 72, or 96 hours thereafter were tested in mice for protective antibodies by titrating dilutions of culture against a constant amount of serum

The cultures of Pneumococcus Type I (S V-I) used in protection tests were of such virulence that the intraperitoneal injection of 10^{-8} cc invariably proved fatal. The protection test was considered negative if 0 2 cc of serum failed to protect 2 of 3 mice against 10^{-6} cc. of culture Sera of rabbits which afforded protection to mice infected with 10^{-6} cc (100 M.L.D) of culture were also tested against 10^{-5} cc. (1000 M L.D) Amounts of culture greater than this were not used

Blood Levels of Free and Acetylated Sulfapyridine—Blood drawn at intervals during the period of sulfapyridine administration indicated satisfactory absorption of the drug The blood levels of free and acetylated sulfapyridine, however, varied greatly in different animals under the same conditions During the period of administration the highest concentration of free drug noted was 15 0 mg per cent, the lowest 0 5 mg per cent The highest concentration of the acetylated compound was 71 2 mg per cent, the lowest 4 3 mg per cent Although the blood level of free sulfapyridine dropped fairly rapidly in the interval between doses and after administration was discontinued, the acetylated form was eliminated much more slowly In order that there should be little or no sulfapyridine remaining in the blood at the time of intradermal infection it was found necessary to discontinue administration of the drug 24 to 36 hours prior to infection Determinations made 24 hours before intradermal infection showed that only 9 of the 34 animals still had free circulating sulfapyridine, although at this time the blood of all of the 22 rabbits tested for the acetylated compound showed its presence in amounts ranging from 1 3 to 71 2 mg per cent

Determinations made immediately before infection upon the blood of 30 immunized rabbits which received sulfapyridine showed the presence of the free drug in 3 instances The blood of only one of these animals contained a measurable quantity (1 6 mg per cent) On the other hand, the acetylated

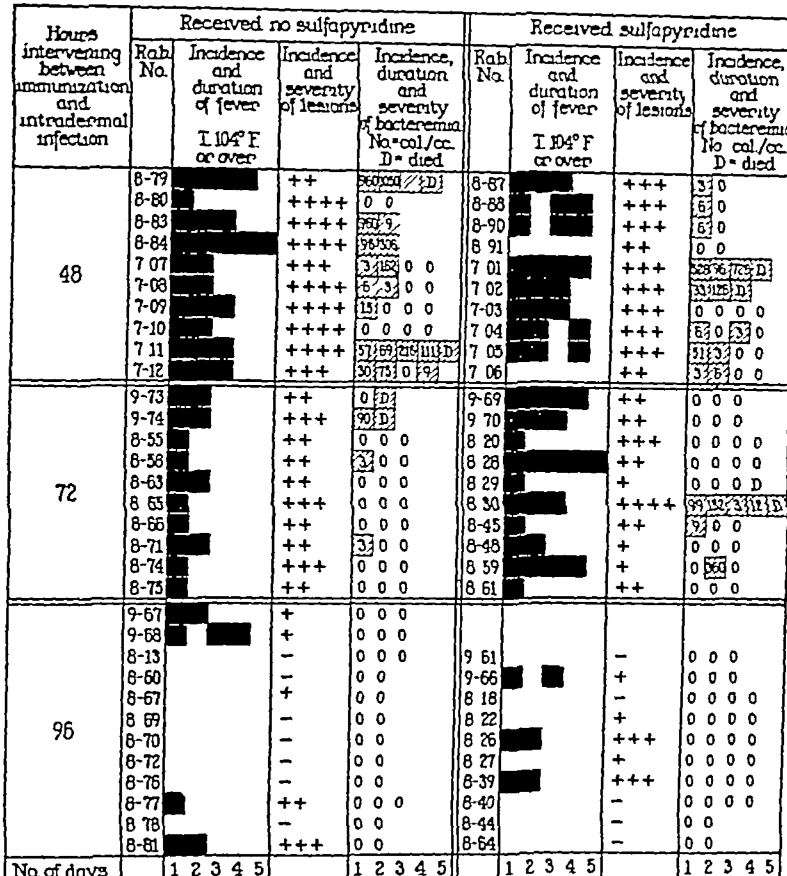
compound was present in the blood of 11 out of 26 rabbits immediately before infection. The levels varied between 0.5 mg. and 5.7 mg. per cent. That these residual amounts of sulfapyridine remaining in the blood at the time of intradermal infection exerted no significant mitigating effect upon the course of the disease is indicated by the following observations. Sulfapyridine was present at the time of intradermal infection in the blood of three of the four treated animals which died. Furthermore, the fatal course of infection in four sulfapyridine treated rabbits which were not immunized, was essentially the same as that in the normal controls. Crystals of acetylated sulfapyridine were found at autopsy in the bladder and renal pelves of two rabbits which developed overwhelming bacteremia.

Toxic Effects of Sulfapyridine.—Almost all of the rabbits which received sulfapyridine showed some evidence of intoxication during the course of drug administration. This was manifested by varying degrees of anorexia, weakness, and loss of weight. Sulfapyridine intoxication may have been a contributory factor in the death of one rabbit which was infected 72 hours after immunization and died on the 4th day after a mild illness unaccompanied by bacteremia. Postmortem examination revealed a loss of 300 gm. in weight, a minimal skin lesion showing evidence of healing and no gross abnormalities of the organs. Analysis of the bladder urine disclosed the presence of acetylated sulfapyridine in a concentration of 2.5 mg. per cent.

It seems evident that the amount of drug used in these experiments was about the maximum that could be tolerated by the animals over the period of administration.

Effects of Intravenous Injection of Pneumococcal Vaccines.—The intravenous injection of heat killed Pneumococcus Type I was frequently followed by an immediate but transient elevation of temperature. No other untoward reactions were noted. The variations of the immune response in sulfapyridine treated and untreated animals at different intervals following immunization are considered later in detail.

Characteristics of the Disease Produced by Intradermal Infection.—The disease produced in rabbits by the intradermal injection of Pneumococcus Type I has been thoroughly described by Goodner (18, 19). The course of the illness in the 26 control animals corresponded closely to his description. The disease was characterized by high sustained fever, extensive dermal lesions, progressive bacteremia, and an invariably fatal termination within 6 days. In immunized rabbits modifications of these findings were progressively more in evidence as the interval between immunization and infection was increased. As a basis for the estimation of active immunity, the effects of infection at different intervals after immunization in rabbits which received sulfapyridine are compared with the reactions of animals to which no drug was given. These data are presented graphically in Text-fig. 1.



TEXT-FIG 1 Characteristics of the disease produced by intradermal infection with Pneumococcus Type I in rabbits previously immunized with a heat-killed suspension of organisms of the homologous type Immunization was carried out by a single intravenous injection of a suspension of heat-killed Pneumococcus Type I equivalent to 10 cc of original culture Intradermal infection was initiated by the injection of 0 2 cc. of a 1 5000 dilution of an 8 to 10 hours rabbit blood broth culture of virulent Pneumococcus Type I. Solid black shading indicates the number of days during which a rabbit's temperature was elevated to 104° or above Absence of shading indicates absence of fever Plus signs represent the occurrence of dermal lesions graded in severity from + to represent a minimal lesion to ++++ indicating one of maximal intensity Minus sign indicates that no lesion developed Cross hatching represents the incidence and duration of bacteremia The small numbers in the cross hatched areas indicate the bacterial colony counts per cubic centimeter Cultures of the blood which proved sterile are indicated by the symbol 0 D indicates animals which died, and with but one exception is placed so as to designate the day of death Rabbit 7-11 died on the 11th day with persisting bacteremia

The criteria for these comparisons include the incidence and duration of fever, the occurrence and severity of the lesions, the degree and duration of bacteremia, as well as the number of fatalities and the time of their occurrence Temperatures of 104°F or above were considered abnormal. The degree of temperature elevation is not included in this report since in most instances high fever continued until the time of death or recovery The severity of lesions has been designated by the symbols + to ++++, to represent the gradations of dermal involvement varying from a small circumscribed tuberculin like reaction to an extensive area of dependent inflammatory edema with ecchymosis and necrosis. The most severe reactions were usually observed in the unvaccinated control animals, but not invariably, since several died before the lesions appeared to be fully developed Evidences of incipient healing of the dermal infection usually paralleled other indications of recovery The rate of healing seemed to depend upon the extent and severity of the lesions The outcome of the infection, however, could not be predicted from the severity of the local inflammatory process, for many of the animals infected 48 hours after vaccination had lesions of maximal intensity but ultimately survived.

In all of the control animals, and with but two exceptions among the 23 vaccinated rabbits which developed bacteremia, cultures of the blood taken 24 hours after infection yielded growth of Pneumococcus Type I. In most of the vaccinated rabbits, notably those with low initial colony counts, cultures of the blood became sterile coincidentally with other evidences of recovery, whereas in some of the vaccinated and in all of the unvaccinated rabbits, bacteremia persisted and progressively increased until the time of death From seven of the eight fatalities among the 62 immunized rabbits, Pneumococcus Type I was isolated in cultures of the blood taken during life or at autopsy The single exception was the rabbit mentioned above which may have died of sulfapyridine intoxication

Non-Immunized Rabbits—In the 26 control animals which received no sulfapyridine the infection followed a uniformly fatal course Included in this number are those rabbits which received 1/10 or 1/100 of the inoculum employed routinely in infecting the vaccinated animals. High fever persisted usually until the time of death and the lesions were characteristically of maximal intensity Cultures of the blood taken 24 hours after infection yielded from three to myriad colonies per cubic centimeter, and in every instance overwhelming blood stream invasion occurred prior to the invariably fatal termination within 6 days In the four unvaccinated rabbits which received sulfapyridine the course and termination of the disease were identical.

Rabbits Infected 48 Hours after Immunization—(Text fig 1) Ten rabbits which received preliminary administration of sulfapyridine and ten which did not were infected 48 hours after immunization. As shown in Text fig 1 all of the untreated rabbits and all but two of those treated with the drug developed

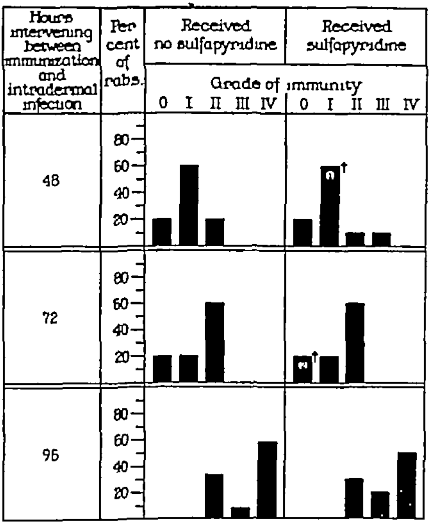
fever which lasted from 1 to 5 days, averaging about 3 days in the febrile animals of both groups The lesions varied in severity from ++ to ++++ in both groups On the whole the intensity of the lesions was perhaps a little less severe in the treated than in the untreated animals However, eight of the rabbits in each group had positive blood cultures 24 hours after infection Although the severity of illness in most of the rabbits was such that death seemed imminent over a period of several days, only two animals from each group ultimately succumbed, three within 4 days and one on the 11th day following persistent bacteremia

Rabbits Infected 72 Hours after Immunization—(Text-fig 1) In the ten treated and ten untreated rabbits which were infected 72 hours after immunization the manifestations of infection were considerably less than in the comparable groups of animals infected 24 hours earlier Although fever occurred in every instance it was of shorter duration, averaging about 1½ days in the untreated and 2½ days in the treated animals The severity of the lesions was also less intense averaging about ++ in both groups with extreme variations of + to ++++ Bacteremia occurred in four of the untreated rabbits of which two died, and in three of the treated animals of which one died One treated rabbit which did not have bacteremia died on the 4th day of illness

Rabbits Infected 96 Hours after Immunization—(Text-fig 1) Half of the rabbits infected 96 hours after immunization, whether treated or untreated, seemed to be unaffected by the same inoculum of Pneumococcus Type I producing severe disease in animals infected after shorter intervals Only four of twelve untreated and three of ten treated rabbits developed fever The average duration was 2 days in each group Lesions occurred in five animals of each group and were for the most part of minimal intensity with individual variations in severity to +++ None of these animals developed bactermia and all survived

Active Immune Response at Different Intervals Following Immunization—In the immunized rabbits manifestations of the disease produced by intradermal infection were progressively less severe as the interval between immunization and infection was increased The enhancement of resistance to infection in animals which had not received sulfapyridine could be explained only on the basis of an immune response to vaccination Likewise, in the animals which received sulfapyridine there was no evidence to contradict this assumption In order to compare the degree of active immunity in the respective treated and untreated groups of animals, and thereby determine whether or not the response in treated animals was affected by drug administration, a modification of the classification devised by Goodner (19) has been employed Immunized rabbits which showed all the characteristics of the disease invariably noted in the controls, including a fatal termination, were considered to have no immunity Those which showed no manifestations of disease were re-

garded as completely immune to the infecting dose employed. Grades of immunity between these extremes have been observed as indicated in Text fig 2 With only two exceptions each rabbit could be readily classified in one of



TEXT FIG 2 Active immunity in rabbits at different intervals following immunization.

Grade of immunity	*Corresponding characteristics of disease*
0 = no immunity	Fever, lesion, bacteremia, and death.
I =	Fever, lesion, bacteremia survived.
II =	Fever and lesion no bacteremia, survived.
III =	Minimal lesion, no fever no bacteremia survived.
IV = complete immunity	No evidence of disease, survived

All of the control animals had 0 immunity

† Only 2 animals did not fit readily into these categories (1) moderate lesion and bacteremia, no fever, survived, (2) fever and lesion, no bacteremia died.

the designated categories Since all of the unvaccinated control animals showed no immunity they have not been included in the chart.

It is evident that as early as 48 hours after immunization most of the treated and untreated rabbits had some degree of resistance to intradermal infection. This is indicated chiefly by the fact that 80 per cent of the animals in both groups survived The immunity was of a low order, however 60 per cent

velopment of circulating antibodies as well as the early development of active resistance is not influenced by the administration of sulfapyridine

DISCUSSION

It is evident from the experimental data that the administration of sulfapyridine has no appreciable effect upon the immune response in rabbits following a single intravenous injection of heat-killed suspension of Pneumococcus Type I

Of particular significance is the fact that active immunity, as indicated by resistance to intradermal infection, was present 48 hours before any appreciable immune response could be detected by tests for circulating antibody 96 hours after vaccination, when antibodies were first demonstrable in the sera, all of the animals tested were almost solidly immune to intradermal infection

Of the techniques employed for the determination of circulating antibody, the mouse protection test was found to be the most sensitive, revealing the presence of antibody in approximately twice as many instances as did the test for agglutinins or precipitins

The precipitin reaction, although an exquisitely sensitive method for the detection of minute amounts of capsular polysaccharide, is less satisfactory than the mouse protection and agglutination tests for the purpose of demonstrating small amounts of antibody As the precipitate which forms the basis of this reaction is composed almost entirely of antibody, small amounts of antibody may fail to produce a visible reaction with varying dilutions of antigen although in the presence of adequate antibody the reaction with antigen in dilution as high as 1 5,000,000 may yield a visible precipitate Furthermore, when used to test the sera of patients with pneumococcal pneumonia the precipitin reaction introduces another possible factor of error If the preparation of capsular polysaccharide employed is contaminated with even a trace of the pneumococcus C polysaccharide the reaction of the latter substance with the C reactive protein of acute phase serum may be misinterpreted as an indication of the presence of type specific antibody (20, 21)

In man studies comparable to those which can be carried out in experimental animals are not feasible Because of the many variables existing in the patient with pneumococcal pneumonia it would be impossible to conclude from the failure to demonstrate circulating type specific antibody that resistance to infection had not developed or that administration of a sulfonamide drug had affected the development of immunity

Either failure to produce sufficient circulating antibody or the removal of free antibody by combination with circulating antigen could preclude the detection of antibody In any event, positive results indicate only that an excess of antibody is present, whereas negative results may signify merely that such an excess cannot be demonstrated by the techniques available

Experimental observations, however, point clearly to the fact that a considerable type specific immune response to pneumococcus develops rapidly and may be adequate to protect against infection in the absence of demonstrable circulating antibody Although the various types of pneumococci vary in their ability to stimulate antibody formation it seems unlikely that the sulfonamide drugs exert different effects upon this particular property in different types of pneumococcus. From the present study it appears that the development of immunity in response to a specific antigenic stimulus proceeds according to the effectiveness of the stimulus irrespective of whether sulfapyridine has been administered.

SUMMARY

1 Sulfapyridine, administered to rabbits during the period of developing immunity after a single intravenous injection of heat killed Pneumococcus Type I, exerted no influence upon the immune response

2 Active immunity as indicated by increased resistance to homologous intradermal infection was present 48 hours after the immunizing injection and 2 days before circulating type specific antibodies were detectable

3 Of the serological techniques employed for the detection of circulating antibody the mouse protective test yielded the highest percentage of positive results followed in order by tests for type specific agglutinins and precipitins, the latter being least satisfactory for the detection of small amounts of antibody

CONCLUSION

The experimental findings lend further support to the view that, in man, effective therapy of pneumococcal infections with sulfonamide drugs is intimately associated with the development of active immunity

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COMPARATIVE DIABETOGENIC ACTION OF THE HYPOPHYSIS FROM VARIOUS ANIMALS

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The rôle of the anterior portion of the hypophysis in the production or intensification of diabetes was discovered in this Institute in 1929 and 1930 and since then has been studied continuously An abstract of this work up to 1935 was published by Houssay in 1936 Some of the extracts of anterior hypophysis in animals without hypophysis and pancreas and with mild diabetes, intensified the hyperglycemia and glycosuria to a degree exceeding the intensity of the diabetes associated with pancreatectomy alone This was observed in the toad (Houssay and Biasotti, 1930), and in the dog (Houssay and Biasotti, 1932, Houssay, Biasotti, and Rietti, 1932, Houssay, Biasotti, Di Benedetti, and Rietti, 1932) These latter observations suggested a possible method of biological assay of the diabetogenic factor The present experiments were undertaken to demonstrate the relative potency of the diabetogenic principle in extracts of the anterior hypophysis from various species.

Test Animals

(*a*) *Dogs* —The hypophysectomized-pancreatectomized dog is difficult to maintain in good condition because the injection of anterior hypophyseal extract profoundly aggravates its condition On the other hand insulin cannot be used since they are so sensitive to this agent they develop fatal hypoglycemia from doses as low as 1 to 3 units per day Normal dogs, while not showing this marked insulin sensitivity require such large amounts of anterior hypophyseal extract (between 1 and 2 gm. per kilo per day for several days) as to prohibit its extensive use for titrating the diabetogenic principle. Mention should be made that this diabetes in normal dogs characterized by hyperglycemia ketonemia hyperlipemia, acidosis etc., (Houssay, 1936) is produced specifically by anterior hypophyseal extracts, extracts of other organs being inert. The hyperglycemia which is preceded and accompanied by increased insulin resistance appears between the 2nd and 5th days after starting with the injections. Upon discontinuing the injections, blood sugar returns to normal in 1 to 3 days. If the animal fasts, the injections produce insulin resistance without hyperglycemia

Dogs with subtotal pancreatic resection demonstrate Sandmeyer's diabetes if less than 3 gm. of pancreas are left This attenuated diabetes is not permanent since it

may either improve or advance progressively to a fatal termination In many cases this diabetes disappears with hypophysectomy if the hyperglycemia and glycosuria are not intense and if the animal is not in a cachectic condition The injection of anterior lobe extract in a dog having Sandmeyer's diabetes rapidly aggravates the diabetic symptoms and results in death (Houssay, Biasotti, and Rietti, 1932)

If 3 to 6 gm of pancreas are left in the dogs, they remain normoglycemic and in good condition, but none the less very sensitive to the diabetogenic action of the anterior hypophyseal lobe extract (Houssay, Biasotti, and Rietti, 1932) We have demonstrated that as the pancreatic tissue is reduced surgically, smaller doses of anterior hypophyseal lobe become necessary The difficulty with the very large pancreatic resections is that the dog may remain permanently diabetic if large or repeated doses of anterior pituitary extract are given In these partially pancreatectomized dogs (retaining 3 to 6 gm of pancreas) with normal glycemia, a permanent diabetes was first obtained with anterior lobe extract This diabetic state persisted in spite of discontinuance of injections of anterior hypophyseal extract and ultimately resulted in cachexia and death (Houssay, Biasotti, and Rietti, 1932) This phenomenon has since been confirmed in the rat by Long (1937, 1939), and in the cat by Dohan and Lukens (1939), and Lukens and Dohan (1940) Young (1937) later made the important observation that when the normal dog was injected with progressively increased doses of anterior hypophyseal extract, diabetes was produced and persisted even though the hypophyseal extract injections were discontinued

The dogs with subtotal pancreatic resection but with normal glycemia are very sensitive to anterior hypophyseal extracts but they present two difficulties (1) permanent hypophyseal diabetes may be obtained in some cases (4 cases of the first 12 dogs studied), (2) very exceptionally hyperglycemia and glycosuria may be obtained from large doses of extracts of tissues other than hypophysis, once in the case of muscle and of thyroid gland in our large report of experiments These two difficulties may be met by (1) leaving at least 4 gm of pancreas, (2) injecting the animals no longer than 4 days, and (3) allowing 7 to 10 days to elapse before beginning a new series of injections

Dogs with extensive pancreatic resection when hypophysectomized, maintain a great sensitivity to the anterior hypophysis extract without showing non-specific diabetogenic reactions When the anterior hypophysis treatment is suspended, the glycemia rapidly returns to the normal level and with few exceptions the dogs recover from diabetes, providing the rules mentioned in the preceding paragraph are followed The animals do not require special care and maintain excellent general health and have been successfully used by Houssay and Biasotti (1938) in their investigations and in the present study

In the normal dog the anterior hypophyseal lobe of the ox has a diabetogenic action From 1932 to the present we have injected this substance into more than 500 animals The bovine anterior lobe showed diabetogenic action in normal animals such as dogs, cats, pigeons, and guinea pigs (listed in order of decreasing action) The action is still less on rats, rabbits, and white mice It has no action on either toads or serpents (Houssay, Biasotti, and Rietti, 1933) According to Young (1938) this action is constant in dogs but inconstant in cats and rabbits Rats, mice, and guinea pigs fail to show a diabetogenic response.

(*b*) *Toads* —In terms of response to the diabetogenic action of hypophyseal extract, the toad deprived of hypophysis and pancreas is the most sensitive animal yet observed However, the animal lives but a short time after removal of the pituitary and pancreas, and there are marked individual and daily variations in response to the injections of anterior hypophyseal extract Comparative experiments must be done in many animals on the same day and under identical conditions with adequate controls Groups of 15 to 20 toads were used in each series.

The diabetogenic action of the hypophysis of different species of animals has been studied in the toad and in other batrachians deprived of the hypophysis and pancreas. In the toad *Bufo arenarum* Hensel the hypophysis of the same toad is diabetogenic (Houssay and Biasotti, 1930) as well as that of fish, chicken, ox, dog and man (Houssay and Biasotti 1931) and also that of *Xenopus laevis*, *Leptodactyllus ocellatus*, and *Bufo paracnemis* Conversely in *Bufo paracnemis* there was a response from *Bufo arenarum* Hensel In the frog, *Leptodactyllus ocellatus* (L) Gir , *Ceratophrys ornata*, and *Bufo d'Orbigny* without hypophysis and pancreas, the hypophysis of the same animal has diabetogenic action In the toad, extracts of other organs of toads and of other species (amphibian bovine or human) are not diabetogenic.

The neurointermediate lobe of the hypophysis of the toad (equivalent histologically and physiologically to mammalian posterior lobe) has a diabetogenic action, although it is less than that of the pars distalis (equivalent histologically and physiologically to mammalian anterior lobe) Likewise the bovine posterior lobe of the hypophysis has some action but it is less active and more toxic than that of the anterior lobe of the same species (Houssay and Biasotti, 1930)

EXPERIMENTAL DATA

The diabetogenic action of the hypophysis from various animals (fish, batrachian, birds, mammals) was studied in normal dogs, in hypophysectomized dogs with 4 gm of pancreas and in toads deprived of their hypophysis and pancreas The diabetogenic action of the serpent hypophysis was not studied in the dog because it had given poor results in the toad, and because it was not possible to obtain sufficient amounts for use in mammals

Experiments on Dogs

Methods —In a few instances normal dogs were injected with human hypophysis in order to verify its diabetogenic action in the animal not deprived of any organ In the remainder of the experiments 9 hypophysectomized male dogs, with pancreas reduced to about 4 gm were used to test the diabetogenic principle

Under ether anesthesia with midline incision partial pancreatectomy leaving the adherent portion to the duodenum with the main excretory duct was first performed. Several days later, when the dog had recovered from the operation total hypophysectomy was done under ether anesthesia using the temporal route. The animals were not injected with hypophyseal substance until they had completely recovered from the surgical procedures and only when they were in excellent general health, usually several months after hypophysectomy Two dogs were used after almost 4 years (Table I)

The dogs were mostly males with an average weight of 10 kilos (from 7 5 to 11 8 kilos) and were kept in metabolism cages and fed raw beef (35 to 40 gm per kilo per day) Fasting blood specimens were taken in the morning preceding the day on which the course of injections was started and on the 4 subsequent days before the injections were given, *i e*, 18 hours after meals and 16 hours after the preceding injection A small cut on the margin of the ear gave the necessary amount (0 2 cc.)

TABLE I

Date of Operations and Sensitivity of Dogs Used

Dog No		Terminal state	Weight	Amount of bovine anterior hypophysis exerting diabetogenic action per day	
				Fresh	Dry
			kg	*mg per kg*	*mg per kg*
5 77	Pancreatectomized 8-4-1937 Hypophysectomized 9-25-1937	Died of diabetes 9-6-1940	10 5	40	9 4
5 78	Pancreatectomized 8-18-1937 Hypophysectomized 9-25-1937	Died of distemper 6-4-1938	9	20	4 7
5-88	Pancreatectomized 11-20-1937 Hypophysectomized 12-15-1937	Died of diabetes 4-2-1938	9 5	40	9 4
5-93	Pancreatectomized 12-31-1937 Hypophysectomized 3-12-1938	Died of distemper 12-4-1939	10	20	4 7
6-03	Pancreatectomized 10-20-1938 Hypophysectomized 11-15-1938	Died of diabetes 12-29-1939	11 8	40	9 4
6-07	Pancreatectomized 10-14-1938 Hypophysectomized 11-21-1938	Alive 6-15-1941	10 1	40*	9 4
6-10	Pancreatectomized 12-16-1938 Hypophysectomized 3-3-1939	Alive 6-15-1941	8 6	20	4 7
6-26	Pancreatectomized 9-27-1939 Hypophysectomized 10-21-1939	Sacrificed 10-16-1940	9 1	40‡	9 4
6-27	Pancreatectomized 9-27-1939 Hypophysectomized 10-21-1939	Sacrificed 10-21-1940	7 5	20‡	4 7

* Later there was not diabetes with 500 mg per kilo per day

‡ Later there was not diabetes with 150 mg per kilo per day

for the Hagedorn-Jensen procedure with deproteinization by the Somogyi technique, for determination of the blood sugar

Hypophyses of various species of animals were used (human, bovine, sheep, dog, rat, guinea pig, chicken, serpent, toad, and fish) The human hypophysis was obtained from the Pathology Department.[1] The other hypophyses were taken from

[1] The authors gratefully acknowledge the courtesy of Professor P I Elizalde and Professor D Brachetto Brian, and of Dr O del Piano and Dr J A. Pique in making the human hypophyseal material available

adult normal animals of both sexes The glands were removed immediately after the death of the animals, except for those of human and fish origin, which were removed a longer period after death and those from chickens which were removed a few hours postmortem. Hypophyses of the rats and guinea pigs were utilized fresh Some were dried on a watch crystal (toad) and others such as those of human beings and dogs were placed in acetone, pulverized after 2 days and then kept in sulfuric acid vacuum. The hypophyses of hens and fish were used as whole glands but those from the other species of animals were used as separate lobes as is noted in each particular case. The glands were triturated in a mortar, suspended in physiological saline solution, and finally stored in the refrigerator until used

TABLE II

Species	Number of glands used	Anterior lobe			Posterior lobe	
		Fresh	Dried	Acetone powdered	Fresh	Dried
		mg	mg	mg	mg	mg
Human beings	10	346	83	66	71	15 3
Dogs	40	43	10	7 09	12.8	3
Bovine	1836	2000	470	435	400	86
Guinea pigs.	3	13.33	2.83	2 60	4.33	0 97
Rats	30	4.57	1 097	0.83	1 43	0.345
Toads	100	3.3	0.55	—	2 1	0 37
		Whole hypophysis				
"Merluza"	6	8 3	1.3			
"Corvina"	6	5 9	0 9			
Chickens	7	6.3	1.3	1 04		

Toad (*Bufo arenarum* Hensel)

The fish 'merluza ' (*Merluccius hubbsi* Marini, 1939)

The fish corvina ' (*Micropogon opercularis* Quoy and Gaimard 1824)

The weight of the whole gland or the weight of its lobes, was determined for the various types used in fresh or dried state and placed in sulfuric acid vacuum for preservation (Table II)

The bovine anterior hypophysis extract was prepared once a week. The gland was removed immediately after the animal was slaughtered and was placed at once in carbon dioxide snow In the laboratory they were kept at a low temperature and the anterior lobe was separated and reduced to pulp with a meat grinder Water (2 400 cc.) and 0 8 per cent sodium hydroxide (600 cc., previously cooled) were added to 800 gm of prepared gland. The flask was shaken continuously while submerged in ice water and then placed in the refrigerator until the next day The mixture was then acidified with 150 cc. of acetic acid (2.5 per cent), then alkalinized with NaOH (0.8 per cent) until a light alkaline reaction to phenol red indicator was observed. After centrifuging the mixture a clear supernatant portion was drawn off and stored in the frozen state in 60 cc. containers.

All injections were given by the peritoneal route twice a day for 4 consecutive days using physiological saline solutions (0 8 per cent) as a vehicle for the hypophyseal substance. The volume used varied in relation to the dose of hypophysis admin

istered but always was less than 5 cc. All possible precautions for asepsis were taken and we have not observed any kind of infection although the sterilization of the extract could not be perfect because of the nature of the material injected

On each test dog the diabetogenic action of the bovine anterior hypophyseal lobe extract was determined by injecting the same dose daily for 4 consecutive days If the fasting blood sugar determination reached 150 mg per cent or more it was considered as a positive result When the test was completed the animal was kept at rest (in normoglycemia) from 7 to 10 days before being used for further determinations If the initial dose was inactive, larger doses were given 4 to 7 days later until a positive result was obtained

After a rest interval the diabetogenic action of the hypophysis of another animal species was determined in the same manner

Results—Bovine Hypophysis The dog's sensitivity to bovine anterior hypophysis was fairly constant (see Table III) A positive result was obtained with 20 mg per kilo per day in 3 dogs and with 40 mg per kilo per day in the other 6 animals From time to time this potency was verified by test It did not change in 3, it increased in 3, the dogs remaining permanently diabetic and in the other 3 a definite decrease of such sensitiveness was observed Not all the anterior hypophyseal extracts have the same activity Nevertheless, it is possible to notice that the dog's sensitivity itself changed little during the long experimental periods (Table III)

The bovine anterior hypophysis produced a diabetogenic action on the dog with its pancreas intact A positive result was observed with 500 mg per kilo per day for 4 consecutive days in 14 per cent of the animals, with 1,000 mg per kilo per day in 53 per cent, and with 1,500 mg per kilo per day in 70 per cent On the hypophysectomized and partially pancreatectomized dogs (with 4 gm of pancreatic tissue) doses of 20 mg of extract per kilo per day in 3 dogs and 40 mg per kilo per day in 6 dogs were sufficient to obtain diabetogenic action In other words, they were 25 to 40 times more sensitive than normal dogs

Human Hypophysis The diabetogenic action of human hypophysis was verified in normal as well as in hypophysectomized and pancreatectomized dogs (with 4 gm of pancreatic tissue left) From autopsy material 170 human hypophyses were assembled, the lobes separated, stored in acetone, and dried An alkaline extract was prepared from this material in powder form by the described technique and placed in the refrigerator

Three normal dogs were injected intraperitoneally In the first, the extract from 59 anterior hypophyseal lobes was given in 4 days, the remaining 2 received the extract from 55 lobes in 2 days The diabetogenic action was definite in the second dog and incomplete in the first and third dogs (Table IV)

In the dogs deprived of their hypophyses and of a part of their pancreas the diabetogenic action was obtained with 13 mg (or a little over that amount) per kilo per day of fresh anterior lobe extract (Table IV) This finding demon-

strates that human hypophysis has a marked diabetogenic action notwithstanding the fact that two unfavorable factors diminished the activity (1) the

TABLE III

Dogs Deprived of Hypophyses and with Only 4 Gm of Pancreatic Tissue Injected Peritoneally Twice a Day for 4 Days with Alkaline Extract of Fresh Anterior Lobe of Bovine Hypophysis (33 Per Cent in Physiological Solution) Blood Sugar Determined from Capillary Blood from Ear Margin, Animals Being without Food for Previous 12 Hours*

Date	Dog No.	Weight	Injected hypophysis per kg per day Weight		Blood sugar Days				
			Fresh	Dry	0	1	2	3	4
		kg	*mg*	*mg*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*
11 23-37	5-77	10 2	20	4 70	26	—	88	127	106
12 26-39	5-77	10 5	20	4 70	67	108	84	62	74
6-27-40	5-77	10.5	20	4 70	93	139	133	196	107
3-10-38	5-77	10	40	9 41	92	—	204	—	255
12 11-39	5-77	10.5	40	9 41	97	147	201	300	—
7- 1-40	5-77	10.5	40	9 41	78	116	177	206	121
11 15-37	5-77	10.3	100	23.52	102	131	—	290	—
10-19-37	5-77	10 6	400	94 08	98	276	—	—	—
	5-78	9	20	4 70	96	—	—	135	—
	5-78		40	9 41	94	—	210	—	225
	5-78		100	23 52	98	106	—	255	—
	5-78		400	94 08	91	—	—	—	280
3-10-39	5-88	9.5	40	9 41	98	—	236	—	272
3-21 38	5-93	10	20	4 70	119	—	—	—	177
3-10-38	5-93	10	100	23.52	102	131	—	290	—
2 3-39	5-93	10	100	23 52	104	105	115	195	280
12 11 39	6-03	11 8	40	9 41	147	207	239	335	—
9-18-39	6-03	11 8	200	47 04	90	—	83	99	—
12-26-39	6-07	10 1	20	4 70	103	101	95	80	119
12-11 39	6-07	10 1	40	9 41	111	138	160	240	—
12 26-39	6-10	8 6	20	4 70	76	172	259	211	247
12-11 39	6-10	8.6	40	9 41	104	238	221	236	—
6-27-40	6-10	8 2	20	4 7	84	122	253	265	—
12-26-39	6-26	9 1	20	4 7	69	106	—	77	92
12 11-39	6-26	9 1	40	9 41	100	93	195	209	—
7 22-40	6-26	11 0	80	18 80	86	91	94	99	89
8- 2-40	6-26	11 0	150	35 25	81	102	104	108	129
12-26-39	6-27	7.3	20	4 7	92	106	—	209	191
12-11-39	6-27	7.5	40	9 41	113	129	138	161	—
7-22-40	6-27	8	40	9 40	79	94	90	89	101
7 29-40	6-27	8.5	80	18 80	88	95	96	104	107
8- 5-40	6-27	8.5	150	35 2	93	103	108	113	138

glands were obtained postmortem from individuals who had been ill, and (2) 6 to 24 hours had elapsed before the gland was available for extract.

Rat Hypophysis The anterior hypophysis of the white rat produced diabetogenic action in doses of 100 glands in 4 days, *i.e.*, 25 glands a day (11 43 mg of fresh glands per kilo per day) on a dog previously responding to 20 mg bovine gland per kilo per day (Table V) There was no action with 40 nor with 20

glands of anterior hypophysis of the rat injected in 4 days The posterior lobe had no action in doses of 40 lobes in 4 days

Dog Hypophysis The hypophysis of the dog showed little diabetogenic activity in our few experiments Diabetogenic action was obtained with 100 glands, but 50 glands injected in 4 days failed to produce any action in 1 dog which had responded to 80 mg per kilo per day of bovine anterior hypophyseal lobe extract (Table V) Because of the few animals used, and also because of

TABLE IV

Normal and Test Dogs Injected with Human Hypophysis

Date	Dog No	Weight	Injected hypophysis per kg per day					12 hr fasting blood sugar				
			No of lobes injected		Weight			Days				
			Anterior lobe	Posterior lobe	Fresh	Dry	Acetone dried and powdered	0	1	2	3	4
1940		*kg*			*mg*	*mg*	*mg*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*
Normal dogs												
6–29	—	6 0	2 36	—	850	200	160	100	98	119	121	110
11–25	—	7 5	3 66	—	1260	300	230	90	112	173	—	—
11–28	—	6 7	4 10	—	1410	340	260	80	79	110	—	—
Dogs with reduced pancreas (4 gm) and without hypophysis												
5–17	6-27	7 5	0 025		8 70	2 09	1 66	84	89	114	87	82
4–15	6-07	10 1	0 026		9 17	2 20	1 75	71	73	102	98	93
5–6	6-07	10 1	0 031		10 48	2 52	2 00	92	97	82	102	81
4-1	5 77	10 5	0 037		13 04	3 13	2 38	80	112	167	235	212
5–6	5-77	10 5	0 037		13 04	3 13	2 38	97	111	177	200	139
4–15	6-10	8 6	0 044		15 25	3 66	2 91	89	170	286	280	239
4–15	6-27	7 5	0 046		15 72	3 77	3 00	74	91	94	100	123
3–11	6-07	10 1	0 075		26 20	6 29	5 00	80	83	147	318	298
3–17	6-26	9 1		0 044	3 15	0 68		76	89	76	69	69
4– 1	6-26	9 1		0 18	12 71	2 74		71	80	83	74	82
3–11	6-10	8 6		5	7 74	1 66		81	95	104	213	265
5–13	6-10	8 6		8	13 50	2 91		92	81	89	98	83

the increasing resistance of the test animal, a final conclusion regarding the minimal active dose cannot be reached On the other hand, in toads without hypophysis and pancreas, the diabetogenic action of the dog hypophysis is extremely intense There was no action with 93 posterior hypophyseal lobes of the dog injected in 4 days

Sheep Hypophysis We have verified the action of the sheep anterior hypophyseal lobe in normal dogs (1,500 mg per kilo per day), but we have not titred it in our partially pancreatectomized animals

Guinea Pig Hypophysis Fresh anterior lobes of 36 guinea pigs (13 3 mg per kilo per day) had no diabetogenic action nor did posterior hypophyseal lobes of 36 guinea pigs (6 0 mg per kilo per day) in dogs sensitive to 150 mg per

kilo per day of bovine hypophysis extract. Larger doses might have been active but were not used

Chicken Hypophysis Diabetogenic action was obtained with 237 whole hypophyses of chickens (35 mg per kilo per day) injected in 4 days. There was a rise of blood sugar to 144 mg per cent with 180 hypophyses of chickens

TABLE V

Dogs without Hypophysis and with About 4 Gm of Pancreas, Injected Intraperitoneally Twice Daily for 4 Days with Hypophysis of Various Animals

Date	Dog No	Weight	Hypophysis injected per kg per day					Blood sugar				
			No of lobes		Weight			Days				
			Anterior lobe	Posterior lobe	Fresh	Dry	Acetone and powdered	0	1	2	3	4
		kg			*mg*	*mg*	*mg*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*
Toad *Bufo arenarum* Hensel (dried pars distalis)												
10- 9-39	6-10	8 6	1 45	—	4 8	0 8		71	102	106	105	115
9- 5-39	6-07	10 1	2 50	—	8 2	1 4		96	107	96	129	131
9-27-39	6-10	8 6	2 90	—	9 6	1 6		112	153	122	146	283
9-22-39	6-10	8 6	5.81	—	19 2	3.2		70	103	182	328	297
Chicken (fresh whole hypophysis)												
2 27-40	5-77	10 5	0 48	—	3 00	0 62	0 495	83	94	96	93	85
3-11-40	5-77	10.5	2.38	—	15 00	3 09	2 47	82	79	92	108	144
4-15-40	5-77	10 5	5 64	—	35 55	7 33	5 87	91	86	133	142	150
Rat (fresh anterior lobe)												
10- 5-38	5-93	10 0	0.50	—	2 28	0.55	—	98	—	102	95	—
12-11-40	6-26	9 1	1 11	—	5 08	1 22	—	86	79	95	82	101
9- 5-38	5-93	10 0	2 50	—	11 43	2 74	—	98	—	—	208	243
3-11-40	6-27	7 5	—	1 33	1 91	0 46	—	76	78	81	74	90
Dog (acetone dried and powdered)												
4-1-40	6-07	10 1	0 40	—	17 02	3 96	4 32	87	80	101	113	82
5-13-40	6-27	7.5	0 90	—	38 70	9 00	8 00	81	92	109	98	93
7 16-40	6-10	8 0	1 59	—	67 0	15 15	10 9	88	97	95	95	106
9-30-40	6-07	9 0	2.77	—	119 0	27 7	15 6	65	82	112	243	286
4-1-40	6-10	8 6	—	0 43	5.58	1.31	1 16	91	89	85	88	81
5-13-40	6-26	9 1	—	0 72	9 24	2 17	2 77	78	83	91	96	81
10-14-40	6-07	9 0	—	2 50	33 0	7 7	5 22	73	84	97	95	97

(15 mg per kilo per day) injected in 4 days in a dog sensitive to 40 mg of bovine anterior hypophysis (Table V) The equivalent diabetogenic potency of chicken hypophysis compared to 20 mg bovine anterior hypophysis was between 7.5 and 15 mg

Toad Hypophysis (Bufo arenarum Hensel) The diabetogenic action of the pars distalis of the toad hypophysis (equivalent to the mammalian anterior lobe) has been studied by Foglia (1940) An intense diabetogenic action was obtained with 200 lobes in 1 animal and with 100 lobes in another injected in 4 days. In 1 dog sensitive to 20 mg per kilo per day of bovine anterior hypoph

However, we hesitate to make this conclusion final since (*a*) the number of experiments was small, (*b*) the test animals showed a rapid loss of sensitivity at the time of the test, and (*c*) the anterior hypophysis of the dog has been found to be very active diabetogenically when tested on the toad

We did not obtain a positive result with either anterior hypophysis of guinea pig or with the whole hypophysis of fish, probably because we did not use sufficient doses The anterior hypophysis of the guinea pig is definitely active on toads and the hypophysis of the fish has a definite action on the same animal although it is inferior compared with that of the other animals studied

The anterior hypophysis of man has been shown to be the most active in partially pancreatectomized dogs as well as in hypophysectomized and pancreatectomized toads But we obtained in addition a positive diabetogenic action in normal dogs with pancreas intact, with a daily dose of 1260 mg per kilo per day of fresh human anterior hypophysis lobe extract, in 2 days time

The diabetogenic power of the anterior hypophyses which were studied can be placed *in the following order of decreasing activity, tested on toads* deprived of their hypophysis and pancreas human, dog, toad (*Bufo arenarum* Hensel), white rat, guinea pig, chicken (whole hypophysis), ox, snakes, "corvina" (*Micropogon opercularis*) We found in the dog a similar order (human, toad, rat, chicken, and ox) The activity of the dog hypophysis on the dog was not established with precision and insufficient amounts of the hypophysis of the guinea pig and "corvina" were injected to demonstrate their action

The diabetogenic action of the posterior lobe was also tested but smaller doses than that of the anterior lobe were injected Nevertheless, it was verified by Houssay and Biasotti (1930 and 1931) on the toad and by Houssay, Biasotti, and Rietti (1932, 1933) on mammals that with equal weights its action is much less intense than that of the anterior lobe Thus 12 7 and 13 5 mg per kilo per day of human posterior hypophysis and 33 mg per kilo per day of posterior hypophyseal lobe of the dog did not produce any diabetogenic action In one instance, the diabetogenic action was obtained with 7 7 mg per kilo per day of human hypophysis in dog No 6-10, but this result was due probably to an abnormal sensitivity of the dog on that date because it was not observed 2 months later in the same dog when 13 5 mg per kilo per day were given

The diabetogenic action of the anterior hypophysis must be considered as specific and peculiar to this organ, as has been demonstrated by Houssay, Biasotti, and Rietti (1932, 1933) because the extracts of other organs did not have such action in 4 days on the same dogs nor 660 mg per kilo per day of the liver or kidney of the toad (including the adrenals), nor 400 to 1000 mg per kilo per day of kidney, liver, spleen, testicle, muscle, and thyroid of dogs or oxen

Neither 30, 40, or 60 mg of corticosterone nor 80 to 200 mg of desoxycorti-

costerone, injected in 2 days produced the diabetogenic effect Houssay and Biasotti (1938) did not obtain any action with prolactin (100 mg in 4 days) or with adrenotropic (adreno-cortico-tropic) extract sent by Collip (250 units), follicle stimulating hormone (F S H) (150 mg), and luteinizing hormone (L H) (100 mg , *i.e* , 100 units)

CONCLUSIONS

Of all the anterior hypophyses tested, those of the human produced the most marked diabetogenic action in the dog with its pancreatic tissue reduced to 4 gm., and in the hypophysectomized and pancreatectomized toad The human hypophysis also produced diabetogenic action in the normal dog on daily doses of 1 26 mg per kilo per day for 2 days

The hypophysectomized dog with its pancreas reduced to 4 gm. is very sensitive to the anterior hypophyseal diabetogenic action and is the best test animal for demonstrating such action in mammals

The anterior hypophysis of man, toad, rat, and chicken produces in such animals a diabetogenic action with doses of from 10 to 15 mg per kilo per day The bovine anterior hypophysis has identical action in 20 mg doses That of canine origin was much less active in a few though inconclusive experiments.

It was impossible to demonstrate a diabetogenic action with either guinea pig hypophysis or with that of fish probably because insufficient doses were injected

The diabetogenic action was not obtained by the injection of other organ extracts of toads, dogs and oxen, of corticosterone (30, 40, and 60 mg in 4 days) or of desoxycorticosterone (80 mg and 200 mg in 4 days)

The toad (*Bufo arenarum* Hensel), deprived of its hypophysis and pancreas is the most sensitive biological reactor for testing the diabetogenic action In this animal the diabetogenic action of anterior hypophyses from varied sources decreased in the following order man, dog, toad (*Bufo arenarum* Hensel), white rat, guinea pig chicken (whole hypophysis), ox, serpent (*Constrictor constrictor* (L)), the fish "corvina" *Micropogon opercularis* (Quoy and Gaimard, 1824), and "merluza" *Merlucius hubbsi* (Marini, 1933)

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any attempt to deduce the natural history of the infection from a given pattern of virus distribution

In 1910, Flexner and Lewis (5) reported that Berkefeld filtrates of the mucous membrane of the nasopharynx obtained from recently paralyzed monkeys produced paralysis in other monkeys In 1911, Landsteiner, Levaditi, and Danulesco (6) reported that a number of attempts to find virus in the nasal secretions of paralyzed monkeys failed until they inserted cotton plugs for 24 hours into the nostrils of 2 paralyzed *cynomolgus* monkeys, and inoculated the pooled filtrate intracerebrally and intraperitoneally into another monkey which developed typical poliomyelitis in 10 days In three tests on the tonsils and peritonsillar mucosa obtained from three paralyzed *cynomolgi*, which were infected by the intracerebral route, one yielded the virus In 1912, Levaditi and Danulesco (7) continued these studies and recorded the following observations (1) tests with filtrates of the nasal mucosa obtained from 4 intracerebrally inoculated, paralyzed *cynomolgus* monkeys were all negative and they stated that similar tests on a larger number of monkeys were also negative, (2) filtrates of the nasal secretions obtained on tampons from 5 intracerebrally inoculated, paralyzed monkeys (2 *rhesus* and 3 *cynomolgus*) yielded negative results when the secretions from each paralyzed monkey were subinoculated separately into a new monkey, (3) when the nasal secretions from two other *cynomolgus* monkeys were pooled and subinoculated into a single monkey, virus was demonstrated, (4) six experiments in which tampons from infected monkeys were placed in the noses of normal monkeys were negative, (5) tests on the tonsils from 4 intracerebrally inoculated, paralyzed monkeys (1 *rhesus* and 3 *cynomolgus*) yielded one positive result with the material from one of the *cynomolgus* monkeys

In more recent years a number of attempts to demonstrate the virus in the nasal or nasopharyngeal mucosa and secretions of paralyzed *rhesus* monkeys have yielded negative results In 1938, Sabin and Olitsky (8) observed that even after nasal instillation of highly potent "M V" virus in *rhesus* monkeys none was found in the nasal mucosa and secretions at the time of paralysis, although virus was demonstrable at 72 hours after instillation by the method used In the same year Sabin (2) reported that no virus was found in the nasal mucosa of 4 *rhesus* monkeys which succumbed with typical poliomyelitis after injection of "M V" virus by the tonsillopharyngeal route In 1939, Kramer, Hoskwith, and Grossman (9) reported negative results when they subinoculated the nasopharyngeal and oral washings together with nasal and pharyngeal tissue obtained from 7 paralyzed *rhesus* monkeys some of which had been inoculated intracerebrally with "M V" virus and others intranasally with the "Armstrong" virus

The early experiments probably differed from those carried out later on in that strains of virus of more recent human origin or *cynomolgus* monkeys were used, and in so far as larger amounts or virus might have been injected intracerebrally and intraperitoneally Theoretically, it is possible that after injection of large amounts of virus intracerebrally some may escape along the known existing connections between the subarachnoid space and the interstitial tissues of the nasal mucosa, although Yoffey and Drinker (10) reported that "M V" virus inoculated intracerebrally or intranasally in *rhesus* monkeys could not be demonstrated in the lymph from the nasopharynx.

In so far as one is searching for corollaries to help explain events in the human disease, it is more relevant to know what happens when the virus has reached the central nervous system from a peripheral site than after intra cerebral injection Fully realizing that ultimately one will have to consider separately the behavior of different strains of virus in different hosts, it was decided to begin the experimental inquiry into the centrifugal spread of polio-myelitis virus with the monkey adapted "M V" strain in *rhesus* monkeys The intrasciatic route of inoculation was selected chiefly because we had reason to expect that poliomyelitis could be produced regularly in this manner, and despite the fact that, according to a report by Hurst (11), virus can some-times be found in the spinal fluid of such monkeys when the disease is fully developed although not in the earlier stages. Since poliomyelitis virus has never been demonstrated in the cerebrospinal fluid of human cases and rarely, if ever, in monkeys inoculated by other peripheral routes, the intrasciatic route of infection might thus perhaps be expected to favor the possibility of escape of virus from the subarachnoid space into the nose

General Plan

The plan of investigation was as follows (1) inoculate "M V" virus into the sciatic nerve of a group of *rhesus* monkeys (2) collect the nasal secretions on absorbent cotton plugs (the method by which successful isolation of virus from intracerebrally inocu lated *cynomolgus* monkeys was reported by Landsteiner Levaditi and Danulesco (6) and by Levaditi and Danulesco (7)) every 24 hours during life (3) pool the secre-tions collected during each 24 or 48 hours from several monkeys and test for virus, (4) when the monkeys were either prostrate or dead as a result of poliomyelitis in fection a number of tissues selected for their capacity to indicate the extent of cen trifugal spread of virus are to be obtained with special precautions to avoid con tamination and tested for virus by subinoculation into new *rhesus* monkeys The spinal cord was included in the group of tissues from each monkey to serve as a posi tive virus control The tests on the olfactory bulbs were expected to indicate not only whether the virus had spread that far but also, in case virus were found in the nasal secretions or mucosa, whether the virus had reached the nose by a neuronal pathway or otherwise. The presence or absence of virus in the tonsils and pharyngeal tissue might be an index either of lymphatic absorption from the nose or centrifugal spread from the medulla Tests on the superior cervical sympathetic ganglia the abdominal sympathetic ganglia of the celiac plexus the adrenals small intestine, and salivary glands were expected to show how far virus localized in the central nervous system wandered into peripheral sympathetic ganglia or tissues containing collections of nerve cells of the parasympathetic system. It is well known that by the time a monkey is prostrate as a result of poliomyelitis infection specific neuronal lesions may be found in almost all the spinal ganglia and also in the sensory cranial ganglia regardless of the route of inoculation (Pette, Demme and Környey (12), Bodian and Howe (3), and personal observations) It is apparent, therefore, that the virus spreads to the sensory neurons close to the spinal cord and medulla and

might thus perhaps be expected to involve also some of the nerve cells in the ganglia of the sympathetic trunk along the vertebral column in the thorax and abdomen although Pette, Demme, and Környey (12) stated that in general no neuronal lesions were found in these ganglia Whether or not it can spread as far as the collateral ganglia, such as the celiac, or the terminal ganglia located within the viscera or glands that they innervate, is a question of considerable importance in any attempt to interpret a given pattern of virus distribution in cases where the original portal of entry of the virus is unknown

Methods

Virus and Mode of Inoculation —The "M V" strain of poliomyelitis virus was used Before each experiment glycerinated cord was passaged and fresh virus was used for intrasciatic injection With the monkey under ether anesthesia an incision through skin and muscle was made midway over the posterior aspect of the thigh and the sciatic nerve was then exposed by blunt dissection 1 cc. of a 10 per cent lightly centrifuged suspension of the virus was then injected into the nerve using a 20 or 22 gauge needle which was moved back and forth within the sheath of the nerve for the purpose of cutting a certain number of nerve fibers and exposing their axis cylinders While ballooning of the nerve occurred during the injection, most of the inoculum escaped into the surrounding tissues It is noteworthy that of 8 monkeys so inoculated all developed poliomyelitis In view of the fact that some investigators (Harrison and Woolpert (13), Toomey (14)) reported that in their experience most monkeys failed to develop poliomyelitis after intrasciatic injection and in view of the fact that it has been suggested that vitamin D deficiency may facilitate progression of poliomyelitis virus along peripheral nerves, it should be pointed out that 6 of the 8 monkeys in the present experiments either had no vitamin D deficiency to begin with or else had been given vitamin D (drisdol) for a period of 2 weeks prior to inoculation, in the other two monkeys both chemical and roentgen evidence of D deficiency were present The relationship between vitamin D nutrition (as determined by the concentration of phosphorus in the blood and roentgen examination of the bones) and the invasiveness of poliomyelitis virus along peripheral nerves was studied on these monkeys but the data will be presented in another communication (15)

Collection and Preparation of Nasal Secretions for Subinoculation —Directly the intrasciatic inoculations were completed, absorbent cotton was plugged into each nasal cavity At the end of 24 hours these plugs were removed and new ones were inserted The moist plugs were kept in a refrigerator or frozen by means of solid CO_2 This process was repeated every 24 hours until the monkey was dead or sacrificed In the first series of tests the cotton plugs for each 24 hours from each of 4 monkeys were pooled for subinoculation into a new monkey In the second series of tests the cotton plugs for each 48 hours from each of 2 monkeys were pooled As much as possible of the original nasal juice was expressed from the cotton plugs and further extracts were obtained by maceration with physiological salt solution following the same procedure of extraction, centrifugation, and etherization that was used in similar tests on human beings and described in a previous communication (16) The untreated (unetherized) centrifuged sediments were instilled intranasally into the same monkeys which received the etherized supernatant liquids intracerebrally and

intraperitoneally Some of the etherized material was saved for a second intracerebral inoculation 4 to 7 days later Several monkeys died of a bacterial pneumonia until the ether anesthesia used for the intracerebral inoculation was replaced by local anesthesia.

Collection and Preparation of Tissues for Subinoculation.—The various tissues were obtained when the paralyzed monkeys were either dead or prostrate. The adrenals, abdominal sympathetic (celiac) ganglia and plexus, the superior cervical sympathetic ganglia, salivary glands (parotid and submaxillary), spinal cord, and olfactory bulbs were all obtained with aseptic precautions and suspensions prepared of them were

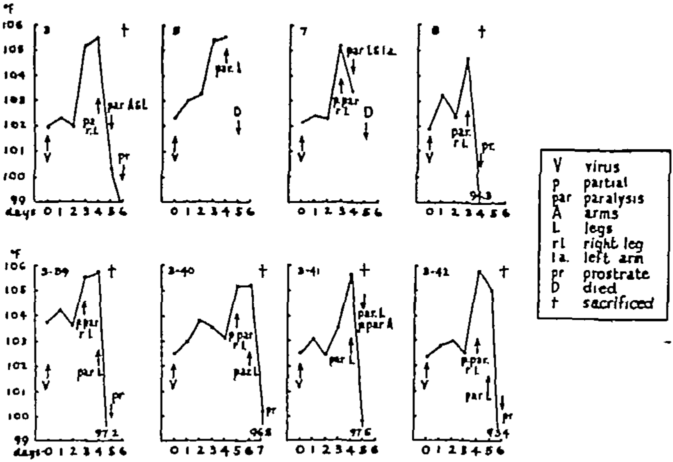

CHART 1 Clinical course of *rhesus* monkeys inoculated into right sciatic nerve.

injected intracerebrally or intracerebrally and intraperitoneally when sufficient material was available. The nasal mucosa (obtained by a method previously described (8)) the tonsils with the attached pharyngeal tissue, and the small intestine were ground with sand and physiological salt solution and the centrifuged supernatant liquids were treated with 15 per cent of anesthetic ether before intracerebral and intraperitoneal inoculation

RESULTS

The clinical course of the experimental disease produced in the 8 monkeys by intrasciatic injection of poliomyelitis virus is shown in Chart 1 It is noteworthy (1) that the incubation period was relatively short, paralysis being observed first on the 3rd day in 3 monkeys, on the 4th day in 4 monkeys, and on the 5th day in 1 monkey, and (2) that the first rise in temperature occurred

TABLE I

Search for Poliomyelitis Virus in Nasal Secretions of Rhesus Monkeys Inoculated with the "M V" Strain

Donor monkeys	Period after inoculation	Test on nasal secretions: Dose and route	Monkey No	Result
	days	*cc.*		
Intrasciatic Rh 3—par 4 Rh 5— " 4 Rh. 7— " 3 Rh 8— " 3	0 to 1	2 i.c. 1.8 i.p. 3 i.n.	18	D_2 (pneumonia)
	1 to 2	2 i.c. 3 i.p. 3 i.n.	19	D_1 (pneumonia)
	2 to 3	2 i.c. 4 i.p. 9 i.n.	20	Remained well—no poliomyelitis
	3 to 4	2 i.c. 3 i.p. 12 i.n.	21	" " " "
	4 to 5	2 i.c. 4 i.p. 9 i.n.	49	D_3 (pneumonia)
Intrasciatic Rh. 3-40—par 5 Rh 3-42— " 4	0 to 2	2 i.c. (× 2) 8 i.n.	3-61	D_1—enteritis
	2 to 4	2 i.c. 8 i.n.	3-62	D_7—enteritis—no poliomyelitis
		2 i.c.	2-82	Remained well " "
	4 to 6 (Rh 3-42) 4 to 7 (Rh 3-40)	2 i.c. (× 2) 10 i.n.	3-63	" " " "
Intrasciatic Rh 3-39—par 3 Rh 3-41— " 4	0 to 2	2 i.c. (× 2) 6 i.n.	2-68	" " " "
	2 to 4	2 i.c. (× 2) 6 i.n.	3-87	D_5—pyogenic meningitis
	4 to 5	2 i.c. (× 2) 6 i.n.	3-88	Remained well—no poliomyelitis
Intracerebral Rh 4—par 5	5 to 7	2 i.c. 0.5 i.p. 6 i.n.	2	" " " "

i.c.—intracerebral, i.c. (× 2)—reinoculated intracerebrally in 4 to 7 days, i.p.—intraperitoneal, i.n.—intranasal, par 4—initial paralysis 4th day after inoculation, D_2—dead 2nd day

on the same day as the paralysis in 6 monkeys and on the day preceding paralysis in 2 monkeys (to be contrasted with the disease following nasal instillation of the virus when the first rise in temperature occurs 3 to 5 days before the onset of paralysis) The appearance of paralysis first in the inocu lated leg of 6 monkeys and in both legs of two others is in accord with the usual course of events The disease was purposely allowed to run its full course

TABLE II

Tests for Centrifugal Spread of Virus in Rhesus Monkeys Succumbing with Poliomyelitis Following Intrasciatic Injection of M V Virus

Tissues tested	Result of test for virus on tissues of donor monkey No.							
	3	5	7	8	3-39	3-40	3-41	3-42
Spinal cord	+	+	+	+	+	+	+	+
Olfactory bulbs	0	0	0	0 [o]	0	0	0	0
Nasal mucosa	0	0	0	0	0	0	0	t.u
Tonsils + pharyngeal tissue	0	0	0	t.u	0	0	0	0
Salivary glands	0	0 [o]	t.u	0	0	0	0	t.u
Small intestine	t.u.	0	t.u	0	0	0	t.u	0
Abdominal sympathetic-celiac ganglia and plexus	t.u	0	0	0	0	0	0	0
Adrenals	0	0	0 [o]	0	0	0	0	0
Superior cervical sympathetic ganglia	0	0	0 [o]	0	0	0	0	t.u

0 [o] refers to monkeys whose nervous tissues were passaged to other monkeys with nega tive results

t.u —test unsatisfactory because monkey died of extraneous causes before poliomyelitis could have developed.

in these monkeys in order to permit the greatest possible spread of the virus

The results of the search for virus in the nasal secretions of these monkeys are shown in Table I. While a number of the subinoculated monkeys died of non poliomyelitic causes, a sufficient number survived in the several series to indicate that no virus is demonstrable in the nasal secretions of the intra sciatically infected *rhesus* monkeys during any stage of the preparalytic or paralytic phases of the disease The tests on the tissues listed in Table II indicate that by the time the terminal phase of the disease is reached or at death the virus had not spread sufficiently either in the central nervous system

to involve the olfactory bulbs and the adjacent nasal mucosa, or peripherally to affect the collateral sympathetic ganglia, such as the superior cervical sympathetic, celiac, or the nerve cells in the adrenals, the collections of nerve cells of the parasympathetic system such as those of Meissner's and Auerbach's plexuses in the small intestine or those in the salivary glands and pharyngeal wall around the tonsils The negative tests with the tonsils offer confirmatory evidence for the absence of virus in the tissue spaces of the nasal mucosa and other regions whose lymphatics drain into these nodes The positive results with the spinal cord of each monkey lend weight to the negative tests with the other tissues

DISCUSSION

The results of the present investigation indicate quite clearly the limited spread of "M V" poliomyelitis virus which has invaded the central nervous system of *rhesus* monkeys by way of a peripheral nerve such as the sciatic While it has been known since Hurst's (11) observations on the histological changes found in intrasciatically inoculated monkeys that the virus invading the lumbar cord rapidly progresses as far as the thalamus and motor cortex, it is now clear that the absence of lesions in the olfactory bulbs of such monkeys, previously noted by Sabin and Olitsky (1) and Bodian and Howe (3), is also associated with an absence of virus It is also noteworthy that no lesions were found in the anterior perforated substance which was examined in 4 of the monkeys used in the present study It is interesting and helpful that this correlation between the presence of specific lesions and occurrence of virus obtains in poliomyelitis, since in most of the other neurotropic viruses studied no such correlation has been found chiefly because there usually is widespread dissemination of the viruses in the central nervous system during the terminal phases of the disease This limited localization of poliomyelitis virus as well as its correlation with the localization of lesions in the central nervous system has recently been found to obtain also in human beings (4)

The negative results with the nasal secretions obtained during various stages of the disease, indicate that there is no justification for the generalization that elimination of virus by the nasal route is one of the consequences of poliomyelitis infection That these negative results with the nasal secretions and nasal mucosa obtained in *rhesus* monkeys infected by a peripheral neural route with "M V" virus are probably not unique is evident from the fact that similar results were recently obtained in studies on human poliomyelitis (4) The direct demonstration by Lennette and Hudson (17) and the indirect indications from the experiments of Armstrong (18) and of Sabin and Olitsky (1), that poliomyelitis virus may be eliminated on the nasal mucosa following intravenous injection of large amounts, apparently have no bearing on the events which take place when infection occurs by peripheral neural routes

The absence of virus in the collateral sympathetic ganglia, the adrenals, the

salivary glands, tonsils and pharyngeal tissue, and the small intestine with its numerous nerve cells of Meissner's and Auerbach's plexuses, is ample evidence against the assumption that, at least in *rhesus* monkeys infected with the "M V" strain, poliomyelitis virus after multiplication in the central nervous system spreads outward again to affect peripheral collections of nerve cells in various tissues The recent tests on the same tissues of human beings with poliomyelitis indicated a similar absence of centrifugal spread, and for that reason the frequent finding of the virus in the human pharynx and intestine suggested these sites as the probable usual portals of entry in man (4)

The possibility that with certain strains of poliomyelitis virus in certain hosts, there may occur a more extensive centrifugal spread cannot be evaluated on the basis of existing data and certainly requires further investigation For instance, in 1914, Flexner, Clark, and Amoss (19) reported two experiments in which virus was demonstrated in the celiac ganglia (*a*) in the first experiment the celiac ganglia from 4 monkeys which died after inoculation with the "K" strain of virus were subinoculated into a *rhesus* monkey which developed clinically and histologically typical poliomyelitis, (*b*) in the second experiment subinoculation of the celiac plexus from a single monkey succumbing to the "K" virus also produced clinically and histologically typical poliomyelitis. The interpretation of these findings in relation to the question of centripetal versus centrifugal spread depends, however, on whether the monkeys from which these ganglia were derived were inoculated only intracerebrally or intraperitoneally as well For if the donor monkeys had been inoculated only by the intracerebral route these findings would indicate a centrifugal spread to the collateral sympathetic (celiac) ganglia, if, on the other hand, they had also received virus intraperitoneally the virus may have been present in the celiac ganglia as a result of centripetal invasion Bodian and Howe (3), for example, observed that lesions in the parasympathetic ciliary ganglia were present in monkeys succumbing after intraocular inoculation ("M V" virus) but not after infection by other routes. Similarly one of us found no lesions in the superior cervical sympathetic and celiac ganglia of 5 *rhesus* monkeys infected intranasally with "M V" virus, while after intraocular injection with the same strain of virus in the same species of monkey Bodian and Howe (3) found occasional lesions in the superior cervical sympathetic ganglia (which contain the sympathetic neurons supplying the eye) in unoperated animals and numerous lesions in monkeys whose ciliary ganglia were removed prior to infection. The recent report of Burnet and Jackson (20) that they found the virus in the sympathetic chain in 2 of 4 *cynomolgus* monkeys which had been inoculated intracerebrally or intraocularly with a strain of virus (Mar) of recent human origin may perhaps be indicative of a certain degree of centrifugal spread to the paravertebral sympathetic ganglia. However, if the sympathetic chain which they tested included the cervical sympathetic ganglia and if the positive results were obtained in the animals which

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were inoculated intraocularly, the virus might have spread there centripetally along the sympathetic fibers and neurons connecting the eye with the central nervous system rather than centrifugally

In view of the high incidence of positive virus isolations from the stools of human beings with poliomyelitis, and the demonstration that the virus is present in the intestinal wall of patients dying of the disease (4), it is of considerable importance to know whether or not poliomyelitis virus can reach the intestinal wall or stools as a result of centrifugal spread from the central nervous system Clark, Roberts, and Preston (21), Howe and Bodian (22), and Toomey (23) reported negative results in tests on the stools of a total of 17 *rhesus* monkeys which were paralyzed as a result of intracerebral inoculation of "M V" virus These negative results are especially significant in view of the demonstration by several investigators that poliomyelitis virus can pass through the alimentary tract of *rhesus* monkeys without being inactivated (21), and the finding in the present investigation that demonstrable amounts of virus do not spread to the intestinal wall from the central nervous system In an attempt to determine whether the same would obtain for a strain of virus of recent human origin and for *cynomolgus* monkeys, we have made some preliminary tests by searching for virus in the colon contents of 3 paralyzed *cynomolgus* monkeys, inoculated intracerebrally with our 1940 "Per" strain, and obtained negative results In 1939, Kramer, Hoskwith, and Grossman (9) reported that they tested separately the contents of the small and large intestines (plus, in each instance, some of the mucosa obtained by curettage) of 7 *rhesus* monkeys some of which received the "M V" virus intracerebrally and others the "Armstrong" virus intranasally While the contents from the large intestine were negative in each case, they found virus in one instance in the contents of the small intestine It would be easier to interpret the significance of this positive finding if we knew whether or not it occurred in a monkey which had received the "Armstrong" virus (presumably a strain of more recent human origin) intranasally (and therefore also swallowed) In work which we have recently completed on *cynomolgus* monkeys which developed paralysis after being fed a strain of virus of recent human origin ("Per") we had no difficulty in demonstrating virus in the wall and contents of the small intestine (24) The possibility that strains of recent human origin may behave similarly in *rhesus* monkeys is now under investigation

SUMMARY AND CONCLUSIONS

1 Eight *rhesus* monkeys with experimental poliomyelitis following intrasciatic inoculation of "M V" virus were used to study the extent of virus spread in the central and peripheral nervous systems and the question of its elimination in the nasal secretions

2 Tests on nasal secretions collected on absorbent cotton plugs daily and

continuously from the moment of inoculation to the end of the disease failed to reveal virus

3 No virus was found in the olfactory bulbs, nasal mucosa, tonsils and adjacent pharyngeal tissue, salivary glands, adrenals, superior cervical sympathetic ganglia, abdominal celiac ganglia, and small intestine

4 Elimination of virus by the nasal route was not one of the consequences of poliomyelitis infection resulting from invasion of the "M V" virus by way of a peripheral nerve in *rhesus* monkeys

5 No indiscriminate widespread dissemination of virus occurred in the central nervous system of the intraneurally inoculated *rhesus* monkeys nor did the virus spread outward sufficiently to involve the collateral sympathetic ganglia or the collections of nerve cells in various peripheral tissues. Under certain circumstances, therefore, the presence of virus in these ganglia and tissues may be used as an index to the portal of entry of the virus

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A COMPARISON OF ERYTHROCYTE SEDIMENTATION RATES AND ELECTROPHORETIC PATTERNS OF NORMAL AND PATHOLOGICAL HUMAN BLOOD

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The suspension stability of blood, as measured by the rate of sedimentation of erythrocytes in plasma, has had wide application in medicine since Fåhraeus (1) published the results of his investigations on the subject some twenty years ago It is well known that the sedimentation rate is increased markedly in many pathological conditions, notably in acute infections However, although a voluminous literature has grown, dealing with many aspects of this subject, the mechanism responsible for the observed differences in the sedimentation rates of normal and pathological blood is not well understood

Various workers have called attention to an apparent correlation between increases in the sedimentation rate and corresponding increases in serum globulin or fibrinogen levels. An excellent survey of the subject was made by Ham and Curtis (2), in which they discussed various techniques for measuring sedimentation rates, the influence of erythrocyte size and total volume on the rate of fall, and correlation with fibrinogen levels

In this paper, we shall report the results of studies on human blood in a number of pathological conditions, obtained by the method of electrophoresis These results have been correlated with corresponding observations on the erythrocyte sedimentation rates.

Materials and Methods

Sedimentation rate measurements were performed on samples of blood[1] taken into tubes containing a dry mixture of potassium and ammonium oxalates, as recommended by Heller and Paul (3) In some cases heparin (Connaught Laboratories) was used as an anticoagulant instead of the oxalate mixture, about 0 1 mg of this material was sufficient for 10 cc. of blood. After thorough but gentle mixing the blood was introduced into Rourke-Ernstene (4) tubes, graduated at 2 mm intervals over a length of 100 mm containing about 1 25 ml. The tubes were suspended in an accurately vertical position in a glass cylinder of water the temperature of which

[1] Fasting samples of blood were drawn from the antecubital vein with as little stasis as possible. The sedimentation rate was measured within 2 hours after collection

remained quite constant, the work being done in a thermostated room at 25°C Readings of a clock were taken as the erythrocyte boundary came to succeeding graduation marks The region of uniform rate of fall determined the (uncorrected) sedimentation rate The partial volume occupied by the cells (hematocrit) was measured after centrifuging the tubes for 30 minutes at 3000 R P M The corrected sedimentation rate[2] was then obtained with the aid of charts published by Rourke and Ernstene (4)

Electrophoretic studies were carried out in the Tiselius apparatus, using the scanning method of Longsworth (5) The technique has been described in detail by Longsworth, Shedlovsky, and MacInnes (6) All the determinations were made in diethylbarbiturate buffer solutions at pH 7 8–7 9 and an ionic strength of 0 05, on samples of plasma or serum which had been diluted with 3 volumes of buffer solution against which they were then dialyzed

RESULTS AND DISCUSSION

In the paper to which we have just referred (6), electrophoretic determinations were made on the plasma or serum from a number of normal individuals, as well as on that of patients suffering from aplastic anemia, rheumatic fever, pneumonia, peritonitis, peritonsillar abscess, acute lymphatic leukemia, lymphogranuloma, obstructive jaundice, lipoid nephrosis, and multiple myeloma The corresponding sedimentation rates were determined by us, but were not published at that time The present electrophoretic studies have extended the work to include more normals and cases of tuberculosis, coronary thrombosis, duodenal ulcer, nephritis, arthritis, lymphosarcoma, burns, fractures, and chemically induced shock in treating insanity The results are summarized in Table I, and some of the corresponding electrophoretic patterns are shown in Figs 1 and 2 In columns 7 to 10 of the table are given the values for the ratios of α globulin, β globulin, γ globulin, and ϕ (fibrinogen) to albumin, respectively The concentrations of albumin in the plasma (or serum) appear in column 5, and the albumin globulin ratios in column 6 The corrected erythrocyte sedimentation rates (E S R) are listed in column 11 The concentrations of the various components were obtained from the areas under the corresponding electrophoretic peaks, such as are shown in Figs 1 and 2 The values found for the normals in the present series agree closely with those found by Longsworth, Shedlovsky, and MacInnes (6) It was pointed out by these authors that the most striking and general change in the electrophoretic patterns of pathological serum is reflected in the α globulin levels, which appear

[2] The rate of fall of particles in a fluid contained in a tube of finite length is determined in part by the partial volume of the particles, since they fall against a countercurrent of the fluid which must rise to replace the space formerly occupied by the particles The counterforce thus exerted against the falling particles depends on the relative volumes of the particles and of the fluid

to be significantly increased in cases of various febrile infections The present results confirm these findings and also indicate that they hold true in cases of

TABLE I

Composition of Normal and Pathological Serum and Plasma

Corrected Erythrocyte Sedimentation Rate

No	Material	Age	Sex	Albumin	A/G	α/A	β/A	γ/A	φ/A	s.r.r.	Temp. a.m.	Remarks
				per cent						*mm./min*	*F*	
1	Normal	9	M	3.45	2.27	0.11	0.18	0.15	—	0.10		
2	Normal	40	M	4.06	1.75	0.16	0.25	0.16	—	0.44		
3	Normal	38	F	4.00	2.00	0.14	0.17	0.19	—	0.50		
4	Oligophrenia	38	F	4.66	1.92	0.11	0.18	0.23	0.10	0.40		Phenyl pyruvic acid
5	Psychopathic personality	22	F	5.05	2.00	0.16	0.12	0.22	0.07	0.30	99.6	During metrazol shock
6	Schizophrenia simplex	16	F	4.33	2.00	0.17	0.14	0.19	0.10	0.55	96.0	During insulin shock
7	Fracture of tibia and fibula	72	M	3.92	2.13	0.17	0.16	0.14	0.11	0.70	98.6	3 days after accident
8	Fracture of femur	40	M	4.21	1.39	0.21	0.30	0.21	—	1.40	101.2	2 days after accident
9	Burns first, second, and third degree	55	F	3.77	1.04	0.36	0.36	0.25	0.14	2.60	101.0	6 days after accident
10	Duodenal ulcer	58	M	4.55	1.56	0.20	0.25	0.19	—	1.20	98.6	Complicated with hemorrhage and obstruction
11	Coronary thrombosis	42	M	3.34	1.07	0.34	0.32	0.27	—	1.40	99.4	1 week after initial attack
12	Coronary thrombosis	43	M	3.40	1.34	0.19	0.30	0.16	—	1.00	98.4	6 weeks after initial attack
13	Tuberculosis	57	M	4.41	1.11	0.19	0.36	0.35	—	1.80	98.6	Chronic pulmonary
14	Tuberculosis	34	M	3.31	0.90	0.34	0.34	0.43	—	1.30	102.0	Bilateral pulmonary far advanced
15	Tuberculosis	39	M	2.95	0.83	0.30	0.34	0.57	0.21	1.80	102.5	Bilateral pulmonary far advanced
16	Tuberculosis	40	M	3.85	1.10	0.17	0.30	0.34	0.10	0.40	100.0	Miliary tuberculous meningitis
17	Tuberculosis	38	M	3.11	1.10	0.33	0.34	0.56	0.24	2.20	100.0	Bilateral pulmonary far advanced
18	Neoplastic disease	36	F	3.80	1.01	0.19	0.29	0.31	—	1.20	98.0	X ray: general skeletal involvement
19	Lymphosarcoma	14	F	3.22	0.70	0.34	0.19	0.91	0.42	1.90	98.0	Febrile Biopsy
20	Chronic nephritis	30	F	3.30	1.33	0.39	0.20	0.26	0.10	1.40	98.4	Toxemia of pregnancy Post partum
21	Arthritis	18	F	3.77	1.19	0.31	0.25	0.26	—	3.00	101.4	Gonococcus, 9 day history

extensive tissue destruction, as evidenced by the patterns for coronary thrombosis, burns, and fractures[3] (Figs 1 and 2) No significant deviation from normal in either sedimentation rate or electrophoretic patterns was found in the cases (4, 5, 6 of Table I) of chemically induced shock in demented patients

Various authors have reported that good correlation exists between sedi

[3] Determinations on the plasmas of three normal individuals whose temperatures were raised by artificial fever to 106 F for 40 minutes showed no increase in α globulin

mentation rates and fibrinogen levels (2) However, the addition of purifie fibrinogen to normal blood fails to increase the rate of sedimentation of ery th

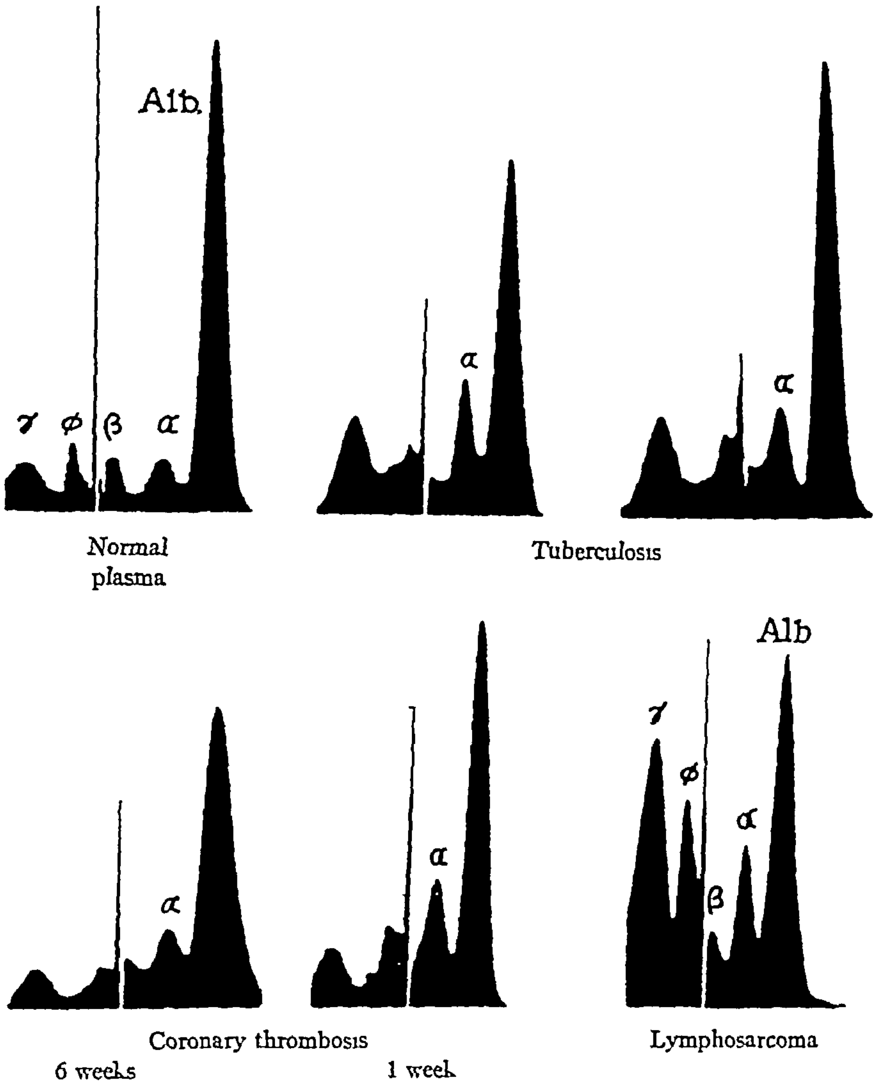

FIG 1

FIGS 1 and 2 Electrophoretic patterns on normal human plasma and on plasma from individuals with tissue injury from various causes

rocytes to the same extent as one observes in pathological blood containing comparable quantites of this protein In Fig 3, we have plotted the sedimentation rate (E S.R) against corresponding levels of fibrinogen expressed as fibrinogen albumin ratios (φ/A), using the results of Ham and Curtis (2)

It will be observed that only a qualitative correlation exists between these two factors

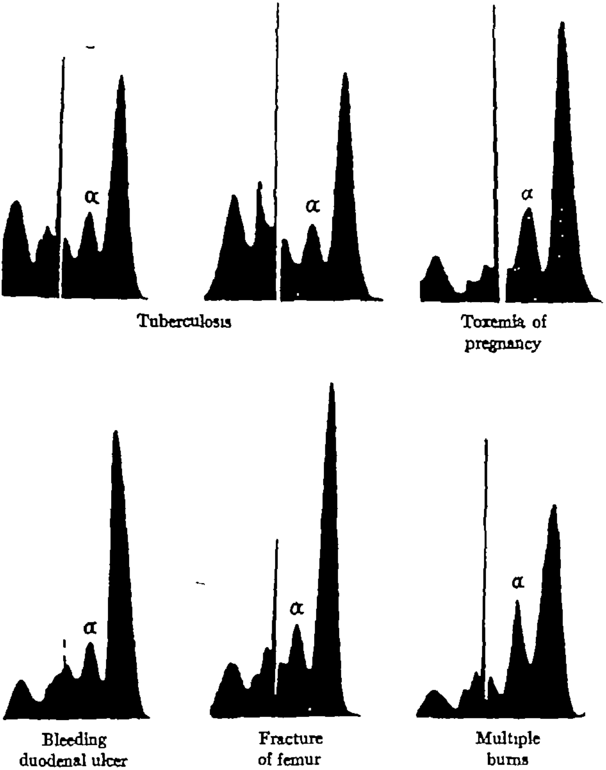

Fig 2

We have found no satisfactory similar correlation with either β globulin, γ globulin, or albumin globulin ratios. However, as will be shown below, a significant correlation can be demonstrated between sedimentation rates and the corresponding α globulin present in the blood.

In Fig 4 are plotted the α globulin levels, expressed as α globulin albumin

ratios, (α/A), against the corresponding corrected sedimentation rates (E.S.R.). In this graph have been included points corresponding to the results reported by Longsworth, Shedlovsky, and MacInnes (6) as well as our more recent determinations. Although it is not possible to draw a smooth curve through the points indicated, any more successfully than in Fig. 3, an in-

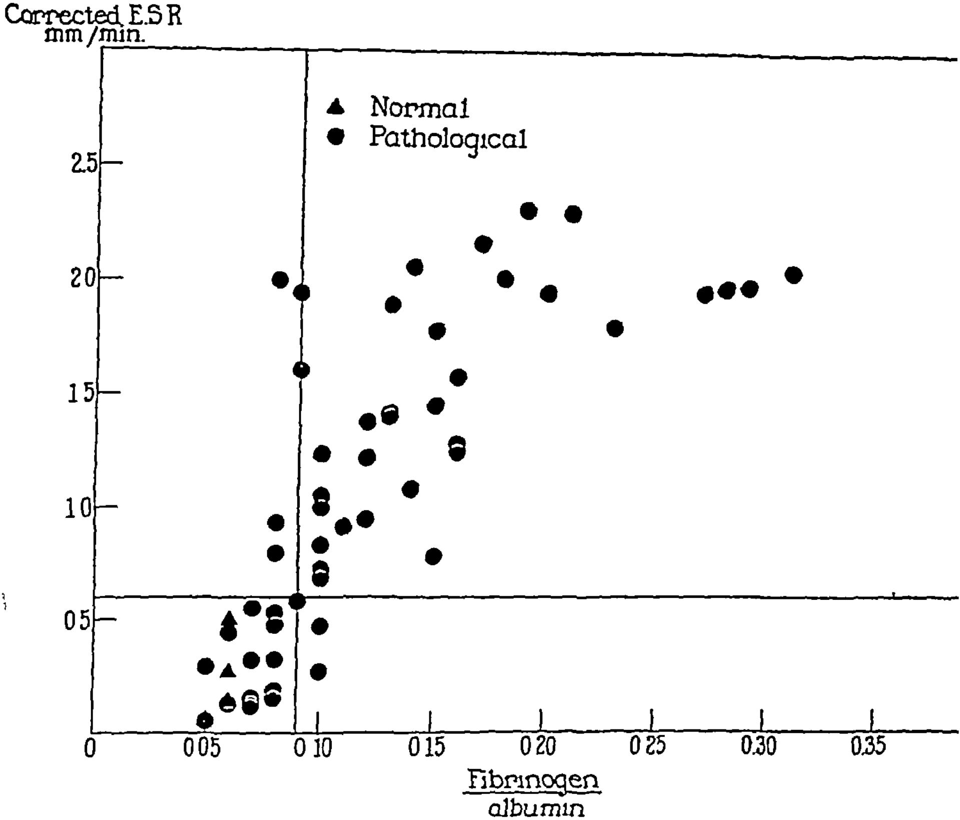

FIG. 3. Variation of corrected erythrocyte sedimentation rates with fibrinogen levels

teresting relationship appears. By drawing a horizontal line corresponding to the upper limit of normal sedimentation rate and a vertical line corresponding to the upper limit of normal α/A values ($\alpha/A = 0.17$), we find all the points corresponding to the normals, as well as those for nearly all the pathological conditions which yield normal electrophoretic patterns, in the lower left hand quadrant. The other points, corresponding to elevated sedimentation rates, fall in the upper right hand quadrant. If we were to draw another vertical line at about $\alpha/A = 0.20$, all the points in this more restricted upper right

hand quadrant, with one exception, correspond to febrile conditions The significance of fibrinogen and of α globulin in the mechanism responsible for increased sedimentation rates in blood will be discussed in another paper

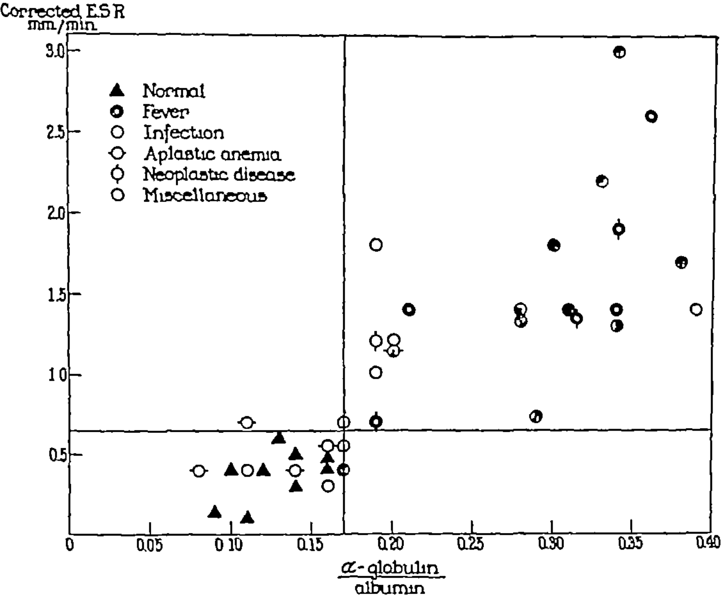

FIG 4 Variation of corrected erythrocyte sedimentation rates with α globulin levels.

We wish to express our appreciation to the following hospitals for their cooperation City of New York Department of Hospitals, Bellevue Hospital, Metropolitan Hospital Presbyterian Hospital, Rockefeller Hospital, Sloane Hospital for Women and State of New York, Department of Mental Hygiene, Psychiatric Institute and Hospital.

SUMMARY

Electrophoretic studies and erythrocyte sedimentation rate measurements were carried out on normal and pathological human blood An increase in α globulin levels appears to take place, as well as an increase in sedimentation

rates, when there is present any considerable inflammation or tissue destruction, irrespective of its cause A graphic correlation is presented between sedimentation rates and α globulin levels, which is at least as good as a similar correlation involving fibrinogen levels

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STUDIES CONCERNING THE SITE OF RENIN FORMATION IN THE KIDNEY

I. The Absence of Renin in the Aglomerular Kidney of the Midshipman Fish

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Plate 1

(Received for publication October 20 1941)

The discovery by Tigerstedt and Bergmann (1) of the pressor substance, renin, in the kidney of the rabbit has been amply confirmed. This substance, moreover, has been found to be present in the kidney of every mammal thus far investigated (2–6) The site of renin production or concentration in the kidney is unknown Goormaghtigh (7–9), on the basis of histological studies of normal and hypertensive kidneys of rabbits and dogs, suggested that renin is secreted by the juxtaglomerular apparatus. On the other hand, Weeks and his associates (10), from their studies concerning the surgical reduction of blood pressure in hypertensive dogs, suggested that the tubule of the kidney is the site of formation of the pressor substance(s) The physiological interdependence of both the glomerular and the tubular vasculature of the mammalian kidney makes the separate assay of either system for renin content difficult However, the reported absence of glomeruli and significant arterial circulation in the kidney of the toadfish (11) suggested that renin assays of both glomerular and aglomerular fish kidneys might provide a unique opportunity for the determination of the intrarenal site of renin production

In the present communication, the results of such a study are reported

Description of Experimental Material

A The Midshipman Fish (*Batrachoididae, Porichthys notatus*) —The midshipman fish was studied because it was suggested to us by Dr Homer W Smith that it might have an aglomerular kidney, as both the midshipman fish and the toadfish belong to the same family (Batrachoididae) Careful histological investigation proved his suggestion to be correct

The midshipman is a marine fish commonly found along the coast of northern California It is about one foot in length and has three peculiar and identifying characteristics (1) when in shallow water it emits a low pitched murmur when irritated or disturbed, (2) it has four body lines consisting of

minute yellow-white circular areas running along its ventral surface, the inner two lines joining each other in a wide circular design, and (3) it has a horny spine at the base of the anterolateral fin and a midline dorsal spine at the anterior termination of the dorsal fin The small circular areas become luminescent at a high pH

The kidney's gross anatomical structure is identical with the kidney of the toadfish as described by Marshall (11) except that no renal arterial circulation and no midline fusion of the separate kidneys were discerned Each kidney weighed about 0 5 gm On histological examination (Fig 1) with the aid of Mallory's triple stain, the kidney parenchyma was observed to be composed of two types of cells,—(1) lymphoid tissue cells and (2) tubular epithelium cells No glomeruli were observed, even on serial section of the entire kidney The tubular epithelium is of one type, consisting of cells which are cuboidal, equipped with a brush border and a nucleus situated proximally in relation to the tubular lumen

B The Carp (*Cyprinidae, Cyprinus carpio*) —The kidney of the carp has been described both by Marshall and Smith (12) and by Moore (13) This kidney receives its arterial blood from the metameric arteries arising from the aorta The glomeruli (Fig 2) are quite numerous, large, and well lobulated At the entrance of the arteriole into the glomerulus, an occasional concentration of cells around the arteriole was observed The nucleus of these cells is vesicular and occasionally surrounded by a halo as described by Kaufmann (14) However, no granules were seen with the Masson trichrome stain, and the exact significance of these cells could not be determined by us The tubular epithelium appears to be of one type and a brush border was present

C The Catfish (*Ameiuridae, Ameiurus nebulosus*) —The kidney of this fish also has been described by the previously mentioned investigators (12, 13) Its vasculature and histological structure were observed to be essentially the same as those of the carp kidney However, the glomeruli did not appear to be as well vascularized and there were fewer juxtaglomerular groups of cells observed in this species of fish

D The Hog —The kidney cortex of the hog was used solely for control purposes The vasculature, glomeruli, and tubular epithelium of the hog kidney were observed to be essentially similar to those of the human kidney

Methods of Extraction and Assay

The extraction of the various types of kidney tissue for renin content was performed with slight modifications according to the method described by Helmer and Page (3)

In brief, 25 to 50 gm of finely ground, fresh or refrigerated (frozen in this laboratory and stored at −40° or −6°) kidneys were dehydrated and defatted by two extractions with ice cold acetone and two extractions with ice cold ether at 0°C The tissue residue was dried in air and pulverized by grinding with sand in a mortar

Crude extracts were prepared by extracting these powders with three separate portions of 2 per cent sodium chloride solution for 1 hour each at 0°C. After centrifuging, the extracts were combined and poured through a coarse filter paper A total of 1 5 cc. of 2 per cent saline solution per gram of fresh kidney was employed but the usual yield after centrifuging and filtering was approximately 1 cc. per gm of tissue. The crude saline extracts were stored at 0°C. until their assay, which took place between 1 and 20 hours after their preparation The pH was adjusted to 7 0 with sodium hydroxide just prior to their injection into dogs.

In certain experiments the extracts were partially purified and concentrated by extending the process to the stage of Fraction B' of Helmer and Page (3) By this procedure, inert protein was eliminated by adjusting the pH of the crude saline extract to 4 5 with acetic acid. After centrifuging, additional inert material was precipitated by bringing the supernatant fluid to 1 M potassium phosphate concentration (pH 6 5) The mixture was centrifuged and the supernatant fluid adjusted to a concentration of 2 M potassium phosphate solution After filtering the residue was dissolved in 6 to 10 cc. of 0 9 per cent sodium chloride solution (Fraction B) All procedures except filtration and centrifugation were carried out at 0°C.

Several portions of the aglomerular kidney were refrigerated at −40°C. for 14 days because of the difficulty in obtaining sufficient material for complete assay experiments. Control storage of hog kidney and carp kidney, at −40 C. and −6°C., indicated that no significant diminution of the renin content occurred at these temperatures during this period of time

Five separate extractions of the midshipman fish kidney (A, B C, D, and E) were performed and the five extracts were tested on three nephrectomized, anesthetized (pentobarbital sodium) dogs. The pressure was obtained by the cannulation and connection of the left femoral artery to a mercury manometer The introduction of hog kidney extract equivalent to 5 gm. of the fresh material into each dog after the injection of aglomerular kidney extract was found to cause a prolonged pressor effect of over 30 mm. of Hg in every dog

Four separate extractions of the carp kidney (A, B C and D) were performed, and the four extracts were tested on five dogs, three of which were anesthetized but not nephrectomized. One extraction of the catfish kidney (A) was performed and tested on two normal anesthetized dogs.

Two separate extractions of hog kidney were performed and tested on two anesthetized dogs (one dog was normal) Also previously tested hog extracts were used for the induction of tachyphylaxis in several experiments.

RESULTS

Despite the fact that five separate extracts of varying quantities of the fresh and refrigerated aglomerular kidneys of the midshipman fish were prepared and tested none of them were observed to exert a pressor effect upon the nephrectomized, anesthetized dog (Table I and Text fig 1) The crude kidney extracts, however, exerted a slight depressor effect on injection

Each of the five extracts of the glomerular kidney of the carp was observed to exert a preliminary depressor and then a prolonged pressor effect upon the

anesthetized dog Greater effects were obtained when the dog was nephrectomized prior to the administration of the extract It should be noted (Table I) that a pressor effect was even obtained with an extract equivalent to as little as 4 5 gm of fresh carp kidney Furthermore, it was observed that after the induction of renin tachyphylaxis by the administration of hog renin

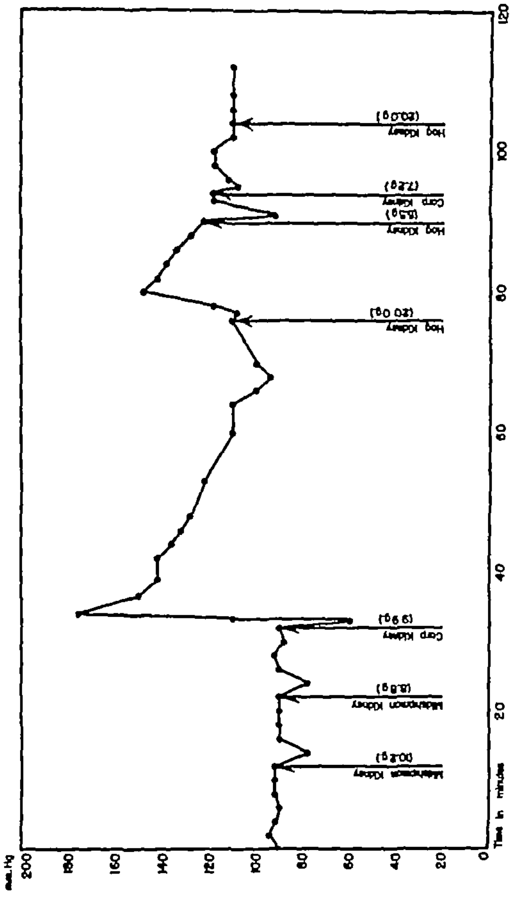
TABLE I

The Pressor Effect of Extracts of the Glomerular and Aglomerular Kidney

Test animal	Type of kidney extracted	Amount of kidney tissue extracted	Pressor response	Duration of pressor effect
		gm	*mm Hg*	*min*
Dog 1	Midshipman fish (A)*	25 0	0	—
Dog 1	" " (B)	32 0	0	—
Dog 2	" " (C)	10 2	0	—
Dog 3	" " (C)	21 4	0	—
Dog 2	" " (D)‡	8 8	0	—
Dog 3	" " (D)‡	20 2	0	—
Dog 3	" " (E)‡	7 5	0	—
Dog 2	Carp (A)	9 9	88	Over 28
Dog 3	" (A)	4 5	30	" 6
Dog 4§	" (B)	8 0	28	" 7
Dog 5§	" (C)*	20 0	20	" 7
Dog 6§	" (D)‖	8 0	48	" 11
Dog 7§	Catfish (A)	5 0	20	" 10
Dog 8§	" (A)	10	40	" 8
Dog 2	Hog (A)**	5 0	60	" 50
Dog 9§	" (B)	5 0	60	" 9

* Extraction carried to "Fraction B" stage
‡ Stored at −40°C for 19 days
§ Dog not nephrectomized
‖ Stored at −6°C. for 12 days
** Stored at −40°C for 14 days

(Text-fig 1), the carp kidney extract failed to exert a pressor effect In one experiment also, the repeated administration of carp kidney extracts (each extract equivalent to 8 0 gm of fresh kidney) was followed by a typical renin tachyphylaxis In view of the fact that (1) carp kidney extract exerts a prolonged pressor effect typical of renin, (2) is capable of producing renin tachyphylaxis, and (3) is incapable of producing a rise in the blood pressure of a dog after the prior induction of renin tachyphylaxis with hog renin, it appears clear that the pressor activity of the carp kidney is due to its renin content



TEXT FIG 1 The pressor effect of aglomerular and glomerular fish kidney extracts upon the nephrectomized dog Also the effect of fish glomerular kidney extract after the induction of *hog renin tachyphylaxis*.

The refrigeration of carp kidney for 14 days at $-6°C$ was not observed to diminish notably its renin content

The extract obtained from 5 0 gm of the glomerular kidney of the catfish also was observed (Table I) to exert a prolonged pressor effect (typical of renin), upon a normal anesthetized dog

The administration of an extract equivalent to 5 gm of fresh hog's kidney was found to exert a prolonged and marked pressor effect when given to a normal anesthetized dog (Table I) Furthermore, refrigeration of hog kidney at $-40°C$ for 14 days did not destroy its renin content

DISCUSSION

The foregoing observations indicate that not only the kidney of the hog, but also the glomerular kidney of the catfish and carp contain renin However, repeated assays of the aglomerular kidney of the midshipman fish failed to reveal the presence of any type of pressor substance Thus, although a strongly pressor extract could be obtained from as little as 4 5 gm of fresh carp kidney, a similar assay performed upon 32 gm of fresh midshipman kidney failed to reveal the presence of a pressor substance In view of these findings, it appears clear that the aglomerular kidney of the latter fish contains no detectable renin

The presence of renin in the glomerular kidneys of both the carp and catfish suggests strongly that the absence of renin in the aglomerular kidney of the midshipman fish is due primarily to the fact that the kidney of this last species lacks the arterial vasculature and glomeruli found in the two kidneys first mentioned It strongly suggests, too, that the site of renin manufacture or concentration is not in the tubules of the mammalian kidney

The presence of a juxtaglomerular accumulation of cells in the kidneys of both the carp and catfish has been described No evidence of the possible endocrine activity of these cells, however, could be detected from our limited histological studies

SUMMARY

1 The absence of glomeruli in the kidney of the midshipman fish (*Porichthys notatus*) is reported

2 The detection of renin in the glomerular kidney of the carp, the catfish, and the hog, and the apparent absence of this substance in the aglomerular kidney of the midshipman fish suggest that the tubular portion of the mammalian kidney does not produce or store renin

The authors wish to express their thanks to Dr Homer W Smith of the New York University and to Dr E K. Marshall, Jr , of Johns Hopkins Medical School who first suggested the use of the midshipman fish Information concerning the fish itself

was given to us by Dr Robert C. Miller of the California Academy of Sciences and by Dr Rolf L. Bolin of the Hopkins Marine Station

We were aided in the histological study of the fish kidneys by Dr G Rusk of the Mount Zion Hospital

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EXPLANATION OF PLATE 1

FIG 1 The aglomerular kidney of the midshipman fish Note the absence of glomeruli, and the presence of an abundant round cell stroma between the tubular constituents Mallory's triple stain × 200

FIG 2 The glomerular kidney of the carp There is an abundant stroma composed of red and white blood cells Note the large and well developed glomerulus Mallory's triple stain × 200

QUANTITATIVE EXPERIMENTS WITH ANTIBODIES TO A SPECIFIC PRECIPITATE

III. Antigenic Properties of Horse Serum Fractions Isolated by Electrophoresis and by Ultracentrifugation*

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The relation of antibodies to each other and to the other serum proteins is a subject of considerable theoretical interest and practical importance Since antibodies are proteins advantage may be taken of a common property of that class of substances—the ability to act as antigens—in carrying out this comparison In the first study of the present series (1) rabbits were injected with a washed specific precipitate composed of the Type II pneumococcus specific polysaccharide and the corresponding anticarbohydrate from a Type II anti pneumococcus horse serum As the polysaccharide is non antigenic in the rabbit (2) this procedure is immunologically equivalent to injecting pure antibody protein The resulting anti antibody rabbit serum was absorbed quantitatively with a variety of specific precipitates derived from horse serum In a second paper (3) the data were extended to include the behavior of both horse and rabbit specific precipitates in immune chicken sera

On the basis of their behavior as antigens the horse antibodies were found to fall into two groups, which corresponded with the immunological classification into antibacterial and antitoxic (prezone) antibodies already noted by Ando (4) The almost identical antigenic properties of the various antibacterial antibodies from horse sera suggested that at least the major portions of the protein molecules must be identical However from this evidence alone it was not possible to decide whether antibacterial antibodies are normally occurring globulins, slightly modified to enable them to react specifically with the appropriate antigen, or whether they constitute a new antigenic fraction not present in normal serum Inasmuch as certain fractions containing antibodies have been isolated from immune sera by ultracentrifugation (5, 6) and by

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

electrophoresis (7) it seemed possible that an immunological study of these and corresponding fractions from normal horse serum might provide further evidence on this point

Methods and Materials

Ultracentrifuge—Most of the preparative and all of the optical runs were made in an air-driven ultracentrifuge (8) kindly placed at our disposal by Dr Aura E Severinghaus of the Department of Anatomy of the College of Physicians and Surgeons Sedimentation constants were determined at room temperatures merely for the purpose of identifying the proteins

Electrophoretic Apparatus—Mobilities were determined in the Tiselius apparatus (9), preferably with the Longsworth scanning method, while the Philpot-Svensson cylindrical lens was found convenient in guiding separation procedures All solutions were dialyzed for at least 2 days in cellophane bags against several changes of buffer at ice box temperatures The completeness of the dialysis was tested in several instances by calculation of the constancy of the refractive index increments of several dilutions of the protein solution with buffer The relative refractive index measurements were made with an interferometer

Anti-Antibody Serum—This was the rabbit serum pool used in the earlier studies and resulted from injection of rabbits with a washed specific precipitate derived by reaction of Type II pneumococcus specific carbohydrate with Type II antipneumococcus horse serum H513[1] Ultracentrifugal and electrophoretic analyses of the serum were carried out, both before and after absorption with polysaccharide

In electrophoresis no definite peak corresponding to a component migrating between the β- and the γ-components was found in the unabsorbed serum as would have been expected from electrophoretic measurements on certain other anti-*Pn* horse sera (7) Measurements were made of the area under the curves, the average ratios of the α-, β-, γ-areas to the albumin area being 0 36, 0 44, and 1 39 for the unabsorbed serum After absorption with the Type II specific polysaccharide the corresponding ratios were 0 34, 0 37, and 0 72 The decrease in the first two ratios is probably within the experimental error, although the association of a small amount of antibody with the β-fraction is not excluded It is apparent, however, that in this particular case most, if not all, of the antibody had the mobility of a γ globulin (*cf* 10) (Table II)

Ultracentrifugal data on a globulin solution from this same bleeding have since been reported elsewhere (11) They indicate[2] that the antibody is quite polydisperse, although most of the antibody had a sedimentation constant of $s = 18 \times 10^{-13}$ cm per second per dyne, there were appreciable amounts with $s = 11$ and 30 (Table I)

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[1] Obtained through the courtesy of Dr A B Wadsworth and Dr H W Lyall of the New York State Department of Health Laboratories

[2] We wish to thank Dr A M Pappenheimer, Jr, for his kindness in sending us the complete data before publication They were made by the Lamm scale method in the Svedberg ultracentrifuge at the University of Wisconsin

Fractions Isolated from Normal and Immune Horse Sera by Ultracentrifugation — Serum from normal unimmunized horses contains a small amount of a high molecular weight component of $s = 18 \times 10^{-13}$ (12) This fraction increases markedly with immunization in certain instances and part of this new globulin has been identified as antibody (5) The rapidly sedimenting protein from two samples of normal horse serum was therefore isolated for comparison with the purified antibody solutions previously investigated (1)

Preparation 1 112 ml. of normal horse serum[3] were diluted 1 1 with saline and and enough merthiolate[4] to give a final concentration of 1 10,000 and run in two portions in an angle ultracentrifuge[5] at 36 000 R P.M for 7½ hours The rotor head was chilled before the run The small amount of jelly at the bottom of the tubes was quickly washed by decantation and redispersed in cold saline The combined solutions were spun down again in the ultracentrifuge Yield about 7 mg of protein Approximate sedimentation constants were determined in the optical ultracentrifuge (Table I) after which the material was redispersed and used in the quantitative precipitation determinations recorded in Table III.

TABLE I

Approximate Sedimentation Constants of Antigen Fractions from Horse Sera

Preparation	Sedimentation constant × 10[13] (cm./sec./dyne)	
	Main component	Other components, in order of concentration
Heavy globulin from normal serum, preparation 1	18	11 5
globulin from normal serum preparation 2a	15	6
" preparation 2b	18	7
Anti Pn II serum H513 antibody containing components[3]	18	11 30
γ-Globulin isolated by electrophoresis of normal serum	8 3	—
γ-Globulin isolated by electrophoresis of immune serum	23	8

Preparation 2 650 ml. of another lot of normal horse serum preserved with 1 10 000 merthiolate were diluted with an equal volume of water and 1350 ml of saturated ammonium sulfate run in drop by drop with stirring. The globulin was allowed to stand overnight, centrifuged, and the precipitate dissolved in water and dialyzed against 0 9 per cent saline The solution was then run in two portions in the ultracentrifuge for 4½ hours at 48 000 R.P.M. The solutions and the centrifuge head were kept in the ice box before the run and the temperature at the end of the run

[3] We are indebted to Dr A J Weil and Dr W G Malcolm of the Lederle Laboratories for the normal horse sera. The horses were in good health at the time of bleeding and had not been used for any immunization experiments

[4] Manufactured by Eli Lilly and Company Indianapolis.

[5] We are indebted to Dr Jacob Furth and Dr Elvin A. Kabat of the Department of Pathology of the Cornell University Medical College for the use of their air-driven ultracentrifuge.

did not exceed 32°C The jelly deposited during the first centrifugation was resuspended in saline and recentrifuged with the second portion Both sediments were again resuspended in saline, merthiolate was added to 1 10,000, and the solutions were allowed to stand for a month to permit complete redispersion An optical run was then made (preparation 2a, Table I) It showed two components, of sedimentation constants s = 18 and s = 7, the former comprising about 50 per cent of the protein A portion of this material was kept for quantitative precipitin determinations The remainder of the solution was again centrifuged in the quantity head at 36,000 R P M for 2⅓ hours The resuspended jelly was then allowed to stand for a month before optical analysis (preparation 2b, Table I) The same two components were present but the heavier material had increased about fourfold An electrophoretic analysis was also performed on this solution (Table II)

Fractions Obtained by Electrophoresis—Two samples of normal horse serum were subjected to prolonged electrophoresis in buffers containing 0 15 M NaCl and 0 02 M phosphate at pH 7 6 In one case it was possible to remove a part of the γ-globulin, in another a small amount of β-globulin (*cf* 13) The γ globulin solution was redialyzed and run again to provide more accurate mobility data, and to verify the electrophoretic homogeneity (Table II) It proved to be quite homogeneous on ultracentrifugation as well (Table I) A portion of the γ globulin was also removed from a bivalent (Types I and II) antipneumococcus horse serum H6225 (Table II) Insufficient material was available to permit a determination of the amount of antibody present, but from the sedimentation photographs it was estimated that about 75 per cent of the protein was rapidly sedimenting γ-Globulin was also isolated from a weak goat Type II antipneumococcus serum, but contained very little antibody A solution of the γ-globulin from normal pig serum was also available [6] It did not react with the antiprecipitate rabbit serum when tested at concentrations up to 0 28 mg N per ml of serum A diluted unfractionated antipneumococcus horse serum was also tested against the rabbit serum

Analytical Procedure—Accurately measured portions of the rabbit antiserum against the horse Type II antipneumococcus specific precipitate were set up at 0°C with varying amounts of the antigens described above The analytical methods were those described previously (1, 14) The tubes containing the precipitates were allowed to stand in the ice box, with occasional mixing, for 48 hours, followed by centrifugation in the cold [7] The precipitates were then washed twice with 3 ml portions of cold saline Nitrogen in the precipitates was determined by a modification of the micro-Kjeldahl method Tests made on the supernatants indicated whether all of the added antigen had precipitated In those instances in which it had, the antibody nitrogen was taken as the difference between the total nitrogen found and the added antigen nitrogen In some cases of incomplete precipitation of the added antigen it was possible to determine the amount of antigen left in the supernatants by setting the latter up with another portion of serum (15) Antibody

[6] Obtained through the courtesy of Dr Elvin A Kabat, now of the Neurological Institute, Columbia University Medical Center

[7] In a refrigerated centrifuge supplied by the International Equipment Company, Boston

nitrogen was then determined by deduction of the corrected antigen value from the total N (Tables III–VII)

Qualitative Inhibition Reactions with Sheep Serum—A portion of the rabbit anti precipitate serum was tested with increasing amounts of unfractionated sheep serum globulin solution A marked precipitate could be obtained at certain dilutions of the antigen, a large excess completely inhibited precipitation Three portions of the rabbit antiserum were then set up with the same excess of sheep globulin One was kept as a control To another was added an equivalent amount of a solution of normal horse globulin, preparation 2b The last portion was treated with an amount of goat γ-globulin solution which gave a good precipitate in the absence of the sheep globulin The tubes were allowed to stand overnight, after which it was found that

TABLE II

Electrophoretic Mobilities of Antigen Fractions in Buffers Containing 0.15 M NaCl and 0.02 M Phosphate

Preparation	Descending mobilities × 10⁵ cm./volt/sec.			pH	Remarks
	α	β	γ		
Ultracentrifuged globulin from normal horse serum, preparation 2b	−2 4	−1 4	−0 7	6 41	
Anti Pn II horse serum H513 unabsorbed	−3 4	−3 0	−0 7	7 55	
Same after absorption with S II	−4 0	−3 2	−0 6	7 55	γ Fraction markedly decreased
γ-Globulin from normal horse serum			−1 3	7 60	Single component
γ-Globulin from immune serum			−0 7	7 49	Single component
γ-Globulin from goat serum			−1 2	7 61	Single component

precipitation had occurred only in the tube containing the added normal horse globulin It was evident therefore that an excess of sheep globulin can completely inhibit the cross-reaction of the rabbit anti-specific precipitate serum with goat γ-globulin There was apparently no inhibition of the homologous reaction

FINDINGS AND DISCUSSION

In recent years much information has become available on the physical properties of horse serum proteins Ultracentrifugal analysis has shown the presence of several globulin components in normal horse serum. The largest in amount is contained in the component with a sedimentation constant, s, of 7, with traces of heavier protein of s = 11, 18, or 32 (12) In antipneumococcus horse serum the concentrations of the heavier fractions, particularly that of s = 18, are markedly increased Most of this new material is anti body (5, 6)

Another type of analysis can be made with the aid of electrophoretic methods. Tiselius has shown (16*a*) that there are at least three globulin components in normal horse serum. Of these, the α-, β-, and γ-globulins are characterized by mobilities of -3.8, -3.1, and -1.0×10^{-5} respectively, in buffers of ionic strength 0.15 at pH 7.7. Tiselius and Kabat have reported (7) the antibody in antipneumococcus horse sera to have a mobility of -1.6 to -1.8 under the same conditions, although some sera showed an additional antibody of lower mobility, -0.3 to -0.9. Moore, van der Scheer, and Wyckoff on the other hand found (10) that all of the antibody in their antipneumococcus sera had a mobility corresponding to that of the γ-globulin. Confirmation of the presence of antibody migrating between the β- and γ-components in other sera has been made however (17).

Relatively few studies have been made on the sedimentation constants of material isolated by electrophoresis, or the mobilities of fractions prepared by ultracentrifugation. Tiselius has reported (16*a*) that horse serum albumin obtained by electrophoresis had the same sedimentation constant as crystalline serum albumin, and that normal horse γ-globulin is also practically homogeneous, with $s = 7.0 \times 10^{-13}$. The α- and β-globulins examined were more complex, in addition to the main components of $s = 6.7$, there were others with $s = 3.1$ and 18 (*cf.* also 16*b*). Electrophoresis of purified antipneumococcus horse antibody solutions which showed polydisperse material in the ultracentrifuge revealed the presence of increased amounts of material of mobility $\mu = -0.3$ to -0.9, as well as the component $\mu = -1.7$ previously identified as antibody (7). The γ-globulin from normal horse serum has recently been shown to be inhomogenous with respect to solubility (18).

In the present study information was desired on the immunological properties of fractions in normal horse serum which corresponded in physical properties with components present in immune sera. It was first established[2] that the major part of the antibody in the horse serum H513, from which the immunizing specific precipitate was derived, was rapidly sedimenting (Table I). The rabbit antiserum to horse anti-Pn II (anti-antibody serum) therefore contained antibody directed against a protein of high molecular weight. Electrophoretic study of H513 showed that the antibody was mainly in the γ-globulin fraction.

Since it has been established that normal horse serum contains a small amount of a high molecular weight component of the same sedimentation constant as antibody (12, 5) it was attempted to isolate enough for comparison with the antigenic properties of antibody fractions. Sera from two normal, unimmunized horses were used. By differential ultracentrifugation it was possible to free most of the rapidly sedimenting material from the lighter components (preparations 1 and 2a, b). The analytical control of preparation 2 was the more complete, and the product was purer, although neither lot was homogeneous. (Tables I and II.)

Both preparations were set up against the rabbit anti antibody serum Preparation 1 removed only about one half of the total antibody present (Table III) Preparation 2a precipitated more of the antibody, and after further ultracentrifugation precipitated practically all of the antibody N from the

TABLE III

Precipitation of Rabbit Anti Pn II Horse Specific Precipitate Serum by Heavy Components of Normal Horse Serum per 1.0 Ml Serum 0°C., 48 Hours

Antigen N added	Antigen N pptd.	Total N pptd.	Antibody N pptd	Antibody N / Antigen N ratio in ppt	Tests on supernatants
mg	mg	mg	mg		
Test antigen ultracentrifuged globulin from normal horse serum, preparation 1					
0 041‡	Total	0 170	0 129	3 1	Excess antibody, no antigen
0 068	"	0 236	0 168	2 5	"
0 095‡	"	0 284	0 189	2 0	"
0 136	0 129§	0 360	0 231	1 8	Slight excess antigen
0 181	0 151§	0 356	0 205	1 4	Excess antigen
Mg. antibody N pptd. = 4.9 (antigen N) − 9 0 (antigen N)[3/2]					
Test antigen ultracentrifuged globulin, preparation 2b					
0 026‖	Total	0 192	0 166	6 4	Excess antibody no antigen
0 034**		0 249	0 215	6 3	"
0 051‡‡	"	0 326	0 275	5 4	
0 102		0 510	0 408	4 0	"
0 153	(0 150)	0 610	(0 460)	(3 1)	Traces antigen and antibody
0 204		0 638§§			Excess antigen no antibody
Mg antibody N pptd = 9 0 (antigen N) − 15.5 (antigen N)[3/2]					

* Assuming all of the antigen N to be precipitated.

‡ Single determination only

§ From analysis of the supernatant.

‖ Quadruple quantities of antigen and serum used for analysis to insure greater accuracy

** Triple quantities used.

‡‡ Double quantities used.

§§ Supernatant gave no precipitate with salt-dissociated Pn I antibody solution.

rabbit serum (preparation 2b, Table III) This indicated that the heavy protein which now constituted the bulk of the preparation was the active antigen, the lighter components in preparation 2a having possibly caused partial inhibition A comparison of the antigenic properties of these materials with other antigens including the salt-dissociated antibody solutions studied previously (1) is given in Fig 1

It is evident that preparations isolated by similar procedures from two different samples of presumably normal horse serum have dissimilar immunological properties In the one instance a product with antigenic properties approaching those of the original pneumococcus anticarbohydrate was obtained This finding raises the question whether a horse with no previous

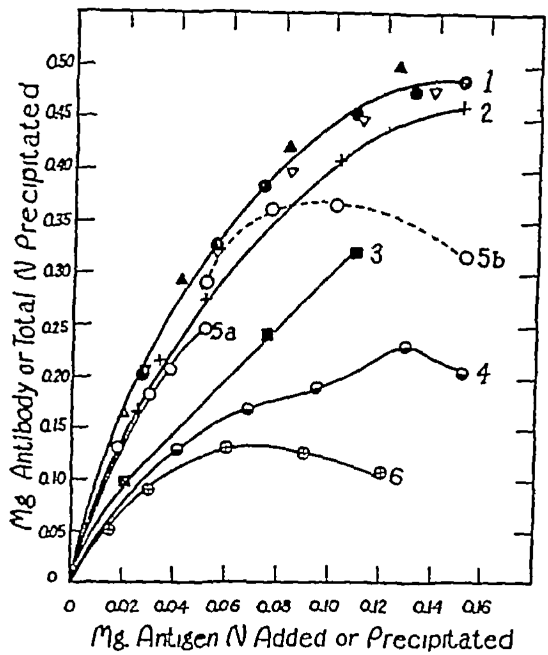

FIG 1 Precipitation of an anti-antibody rabbit serum by various antigens Curve 1, ● = Pn I, II horse γ-globulin, ▲ = Pn II salt-dissociated antibody solution (1), ▽ = Pn I salt-dissociated antibody solution (1), Curve 2 = normal horse heavy globulin, preparation 2b, Curve 3 (total N precipitated) = normal horse β globulin, Curve 4 = normal horse heavy globulin, preparation 1, Curve 5a = normal horse γ-globulin, Curve 5b (total N precipitated) = normal horse γ-globulin, Curve 6 (total N precipitated) = goat γ-globulin

history of immunization might not at some time have been subjected to an unrecognized infection sufficient to induce antibody formation The problem could only be solved by extensive experiments

Less uncertain are the differences in antigenic behavior between a normal γ-globulin and γ-globulin containing antibody (Table IV) In this instance two serum proteins which have the same electrophoretic mobility and are homogeneous by that physical criterion can be distinguished from each other by a second physical method, ultracentrifugation, and by the immunological

method (Tables I, II, IV, and Fig 1) An interpretation of this behavior in terms of the quantitative reaction constants is given below

A sample of the low molecular weight β-globulin from normal horse serum also cross-reacted with the anti-specific precipitate rabbit serum (Table V)

TABLE IV

Precipitation of Antibody from Rabbit Anti Pn II Horse Specific Precipitate Serum by Fractions Isolated by Electrophoresis from Normal and Immune Horse Sera
per 1.0 Ml Serum 0°C, 48 Hours

Antigen N added	Antigen N pptd	Total N pptd.	Antibody N pptd	Antibody N / Antigen N ratio in ppt.	Tests on supernatants
mg	*mg*	*mg*	*mg*		
Test antigen γ-globulin solution from normal horse serum					
0 018‡	Total	0 149	0 131	7 3	Excess antibody no antigen
0 030‡		0 213	0 183	6 1	"
0 038§		0 243	0 205	5 4	" "
0 051‖		0 296	0 245	4 8	" "
0 076	Incomplete	0 354			Both antibody and antigen
0 101‖		0 356			" "
0 152	"	0 306			" "
Mg antibody N pptd. = 10.8 (antigen N) − 26.6 (antigen N)$^{3/2}$					
Test antigen γ-globulin solution from an antipneumococcus horse serum					
0 027§	Total	0 229	0 202	7 5	Excess antibody, no antigen
0 055‖	Total	0 381	0 326	5 9	" "
0 073‖		0 456	0 383	5 2	" "
0 110	0 108**	0 562	0 454	4 2	Excess antibody trace antigen
0 137	0 131**	0 606	0 475	3 6	Trace antibody and antigen
Mg antibody N pptd. = 10.8 (antigen N) − 19.8 (antigen N)$^{3/2}$					

* Assuming that all of the added antigen is precipitated.

‡ 2.5 times *this quantity of antigen and serum* used for analysis.

§ Double quantities used.

‖ One and one half quantities used.

** From analysis of supernatant.

A complete curve could not be constructed as the sample was antigenically inhomogeneous, but the data differentiate it clearly from the antibody containing fractions. An attempt was also made to study the immunological behavior of antibody migrating faster than the γ-component, but in two sera which contained appreciable amounts of this component a portion of the antibody occurred in the γ-globulin fraction as well (*cf* 6) A specific precipitate

made from one of these sera had practically the same quantitative properties as the γ-antibody specific precipitates (1) Another form of antipneumococcus horse antibody having a molecular weight of 150,000 has recently been reported (11) No samples were available

Marrack and Duff (19) concluded that a whole globulin solution from normal horse serum removed only a portion of the antibody from anti-specific precipitate serum However, when we used whole antipneumococcus horse serum as antigen the total N precipitated corresponded to that expected from the antibody content of the serum (*cf* 4)

It will be noted from Fig 1 that antipneumococcus Types I and II horse antibodies isolated by salt dissociation and used as antigens follow closely the curve for the γ-globulin from an antipneumococcus horse serum The data may be expressed by an equation which has been found to represent satis-

TABLE V

Precipitation of Antibody from Rabbit Anti-Pn II Horse Specific Precipitate Serum with Normal Horse β-Globulin per 1 0 Ml Serum, 0°C, 48 Hours

Antigen N added	Antigen N pptd	Total N pptd	Antibody N pptd	Tests on supernatants
mg	*mg*	*mg*	*mg*	
0 020	Total	0 10	0 08	Excess A, no antigen
0 072	Incomplete	0 24	(0 17)	Excess antigen, no A
0 109	"	0 32	(0 22)	Excess antigen, no A

Single determinations only

factorily the behavior of a number of other protein-antiprotein systems (20, 21, 1)

$$\text{mg antibody N precipitated} = k_1 \text{ (antigen)} - k_2 \text{ (antigen)}^{3/2} \qquad (1)$$

This equation must be regarded as empirical, for it is not yet possible to give the constants definite chemical and immunological significance as is the case with the second power relation derived for a number of other systems (14, 21) Several conclusions may be drawn, however, when reactions show identical constants The variation in the constants for the reactions of the γ-globulin solution (Table IV) and the salt-dissociated antibody solutions (1) are within the limits of experimental error This would indicate that no significant change in antigenic properties had occurred during the chemical treatments with salt The somewhat lower constants for the reaction of the heavy normal globulin, preparation 2b, (Table III) are probably due to other antigens of lesser reactivity still remaining (see p 141)

If the equations for the above are compared with that for the reactivity of the normal horse γ-globulin with the same anti-antibody serum it will be noted (Table IV) that the decrease in precipitating power of the normal globulin to

about one-half of the others is reflected in the different reaction constants obtained If, however, the equations for the normal and immune γ-globulins are recalculated[8] to the same maximum of 1.0 mg of antibody N removed, the constants of the equations become identical (Table VII), the equation being expressed as

$$\text{mg. antibody N precipitated} = 10.8\,(\text{Gb N}) - 13\,6\,(\text{Gb N})^{3/2} \qquad (2)$$

where Gb N is the amount of normal or immune γ-globulin nitrogen The reactivity of the normal horse γ-globulin *per mg of antibody precipitated* is therefore the same as that of the immune γ-globulin antigen, and it is evident that the portion of the rabbit antibody which is precipitated by both globulins is incapable of distinguishing between them The remaining portion of the antibody precipitates only the immune globulins or the heavy normal horse protein These findings may be compared with others from this laboratory, in which it was noted that a cross-reacting antibody solution reacted equally well with two different pneumococcus specific polysaccharides (22) or that serum supernatants from the absorption with heterologous polysaccharide (23) or specific precipitate (1) still yielded the same reaction curve with the homologous antigen as did the original serum, when calculated to the same antibody content

The following hypothesis may be advanced to account for the cross reaction of normal horse γ-globulin with antisera to the γ-globulin from immune sera The chemical composition of antibodies and normal globulins from the same species appears to be quite similar (24) The striking differences between the two γ-globulins are the sedimentation constants and therefore the molecular weights, approximately 150,000 and 910,000 If the large size of the immune γ-globulin were due to a polymerization of six specifically altered γ-globulin units, it might account for the cross-reaction of a single normal γ-globulin unit with antiserum toward the polymerized molecule Such specific groups on the antibody γ-globulin molecule as are responsible for its function as an antibody need not be taken into account here since they apparently are not antigenic (1, 3)

If this view were correct, the cross-reaction of the normal horse γ globulin with an antiserum to the larger immune γ-globulin would be analogous to the precipitation of horse antisera to undegraded pneumococcus carbohydrate by various degraded pneumococcus polysaccharides (25) In Table VII are given

[8] The amount of antigen required to precipitate the calculated maximum amount of antibody, A may be found by differentiating equation (1) with respect to the antigen and equating to zero (*cf* 21) Specifically equation (2) becomes $10\,8 - 3/2 \times 13\,6\,(\text{Gb N})^{\frac{1}{2}} = 0$, and is solved for Gb N Substitution of this new antigen value in equation (1) gives the corresponding maximum A Conversion to a basis of 1 0 mg A may be made by multiplying the slope, k_1, by the $\sqrt{A}$ calculated above.

data on the reaction of a number of Type III pneumococcus polysaccharides which had been partially hydrolyzed by acids This treatment is known to break down the polysaccharide chain into shorter units (25) and can be followed by changes in the viscosity or in the amount of reducing groups It will be noted that in spite of the smaller quantities of antibody precipitated by a given amount of the acid-treated polysaccharides, the reaction constants calculated to a common maximum amount of antibody precipitated (1 0 mg N) are remarkably similar to each other and to those for the untreated material In contrast is the behavior of the Type I pneumococcus polysaccharide treated with alkali Under these conditions changes in the polysaccharide are known to be more complex than simple depolymerization (2*b*, 26) The data available, while less numerous, are quite concordant and show clearly the marked differences in the reaction curves produced by the action of alkali on this antigen

TABLE VI

Cross Reaction of γ-Globulin from Goat Serum with Antibody from Rabbit Anti-Pn II Horse Specific Precipitate Serum per 1 0 Ml Serum, 0°C, 48 Hours

Antigen N added	Antigen N pptd	Total N pptd	Antibody N pptd	Antibody N / Antigen N in ppt *	Tests on supernatants
mg	*mg*	*mg*	*mg*		
		Test antigen γ-globulin of goat serum			
0 015‡	Total	0 052	0 037	2 5	Excess A, no antigen
0 030‡	Incomplete	0 092			Slight amount antigen and antibody
0 060§	"	0 132			Excess antigen, trace antibody
0 090	"	0 126			Excess antigen
0 120§	"	0 108			" "

* Assuming all of the added N to be precipitated

‡ Double quantities of antigen and serum actually used

§ Single determination only

A slight cross-reaction with the anti-antibody rabbit serum was observed for the isolated γ-globulin from goat serum (Table VI) No sedimentation data were available for this preparation Comparison could be made, however, of the relative precipitating powers of two antigenic fractions from two different species toward antisera to one of them From Tables III and VI it may be estimated that the goat γ-globulin removed about 15 per cent of the antibody toward the homologous (horse) antibody γ-globulin A preliminary analysis of the cross-reactivity of electrophoretically isolated goat albumin against a rabbit antiserum to crystalline horse serum albumin showed that the antibody N precipitated in the equivalence zone was about 10 per cent of

that removed by the horse albumin [9] The order of magnitude in the cross reactions between goat antigens and rabbit antisera to the corresponding fractions from horse sera is thus the same

TABLE VII

Precipitin Reaction Characteristics with Antibody N Precipitated at One Antigen Level, and Empirical Reaction Constants Calculated to a Maximum of 1.0 Mg of Precipitable Antibody

Preparation	Antibody N pptd. per ml by given amount of antigen (experimental)	Reaction constants equation (1) per 1.0 mg maximum antibody: Intercept, k_1	Slope k_2
	mg		
Rabbit anti-specific precipitate serum with horse globulin fractions			
	0.05 mg protein antigen N		
Pn II antibody solution (1)	0 320	10 9	13 9
Pn I antibody solution (1)	0 305	9 9	11 9
Antibody γ-globulin	0 310	10 8	13 6
Normal γ-globulin	0 240	10 8	13 6
Normal heavy globulin, preparation 1	0 145	4 9	4 1
" " preparation 2b	0 270	9 0	10 3
Horse anti Pn III antibody solution and various S III preparations*			
	0.15 mg S III		
Untreated S III	2 12	39 5	95
S III HCl A	1 90	40 0	98
S III, H_2SO_4 B	1 68	41 4	102
S III HCl C	1 62	40 7	100
S III, HCl D	1 36	39 5	96
S III H_2SO_4 C	1 30	43 5‡ / 37 0	111‡ / 87
Horse anti Pn I serum and S I preparations§			
	0.15 mg S I		
S I untreated	0 340	12 8	17 6
S I, treated with alkali at 37°	0 220	10 7	13 5

* Calculated from (25) and unpublished experiments in this laboratory by Dr Forrest E. Kendall. The fractions are described in (25)

‡ Because of the scattering of the points about a straight line the highest and lowest values calculated from the data are given.

§ From unpublished data by Mrs. H. F Havas.

The technique most commonly used in determining the degree of zoological relationships is to inject rabbits with the whole serum of one species and to test

[9] The serum used was calibrated by Mr Manfred Mayer of this laboratory

the resulting antiserum with whole serum from another species Interpretation of the results is complicated by a number of factors Whole serum is a mixture of antigens which may differ considerably in their relative concentrations and in their antigenic powers An undue proportion of the antibodies produced might therefore be directed toward a minor serum constituent If this complicated mixture of antigens and their antibodies is examined by the usual dilution methods multiple zones of precipitation often result, with partial or complete inhibition of some of the reacting systems The use of dilution methods and whole serum would, for example, completely obscure the relation between the antigenic properties of the normal horse γ-globulin and the immune horse γ-globulins traced above

A more direct method would be to study the antigenic properties of one or more homogeneous fractions isolated by chemical methods or by electrophoresis or ultracentrifugation from the sera to be compared The interaction between these antigens and their antibodies could then be satisfactorily determined by quantitative absolute methods, such as have already proved useful in comparing the species specificities of mammalian thyroglobulins (27) Further studies along these lines would show whether the percentage of cross-reaction of various serum fractions is constant for related species (as in the instance of the horse and goat albumins and γ-globulins here reported) or variable, with perhaps limiting cases in which only the albumins (or globulins) possessed groupings in common Whatever the findings, it is possible that this information would be of more fundamental significance for general physiology as well as zoological classification than are the conclusions drawn from the average behavior of complicated mixtures such as whole sera

It is concluded from the data presented that antipneumococcus antibodies from horse sera resemble in antigenic behavior certain normally occurring horse serum globulins, while they differ in some respects from others having the same sedimentation constant or electrophoretic mobility Thus the antibodies were practically identical in antigenic properties with a rapidly sedimenting globulin from one sample of supposedly normal horse serum but differed in these properties from a sample with the same sedimentation constant from another horse Differences in antigenic behavior between the normal and immune γ-globulin of the same electrophoretic mobility were accompanied by differences in sedimentation constants and molecular weights, in accord with quantitative relations among the immune properties suggesting that the differences are but reflections of the variation in chain length

SUMMARY

1 Rabbit antisera to a Type II pneumococcus specific precipitate from horse serum were tested with fractions prepared by ultracentrifugation and electrophoresis of normal and immune horse serum

2 In one instance a rapidly sedimenting protein from normal horse serum

had nearly the same quantitative antigenic properties toward the anti-anti body rabbit serum as did the purified pneumococcus antibody solutions previously reported In another instance a comparable fraction removed only a part of the rabbit antibody

3 Electrophoretic γ-globulin from an immune horse serum had quantitatively the same antigenic properties as did antibody solutions prepared by salt-dissociation of specific precipitates.

4 Electrophoretic γ-globulin from normal horse serum differed in its antigenic behavior from γ-globulin containing antibody The data are compared with the antigenic properties of acid and alkali treated pneumococcus specific polysaccharides toward antipneumococcus horse sera An interpretation in terms of polymers is suggested

5 The cross-reaction of goat serum γ-globulin against the anti-antibody serum is reported and the extent of the reaction compared with those of goat and horse serum albumins against a rabbit antiserum to the latter

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THE LS-ANTIGEN OF VACCINIA

I. Inhibition of L- and S-Antibodies by Substances in Treated Vaccine Dermal Filtrate*

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A relationship between the heat labile (L) and heat stable (S) soluble antigens of vaccinia has been shown to exist by several workers. Craigie and Wishart (1) originally regarded the two antigens as occurring in the form of a complex which elicited two separate antibodies in animals. They were led to this conclusion because both antigens regularly are found in equal amounts in filtrates of infected tissue and because absorption with either specific antibody removes both serologically active substances. Parker (2) observed that under certain conditions different results were obtained, *e.g.*, a solution containing both precipitable substances when treated with S-antibody lost its S-antigen but retained some L-antigen. Craigie and Wishart (3), at about this time, made similar observations, they reaffirmed that L-antigen ordinarily occurs in a state of combination with S-antigen but reported that with prolonged storage in the cold "this combination may dissociate into separate L and S fractions prior to ultimate inactivation of the L antigen." The data of Parker and of Craigie and Wishart that suggested dissociation of L- and S-antigens have not been duplicated in our laboratory. This failure we believe indicates that conditions for successful repetition of such experiments occur infrequently. That they do occur occasionally we have no doubt, for, as will be shown in a subsequent paper (4), a solution with L-activity only can be prepared from pure LS-antigen by enzymatic digestion.

In our experience, L-antigen has always been encountered in association with S-antigen except under the special circumstances just mentioned. This was true even though at various times during the past five years we have thought of the L-reacting substance as a protein, a carbohydrate, a fat, or a polypeptide and have attempted to isolate such an hypothetical material from dermal filtrates by appropriate methods. When it became evident that the methods employed were inadequate to procure preparations of L-antigen which were free of S-antigen for comparison with the relatively pure S-protein of Parker

* Presented in New Haven before the Society of American Bacteriologists, December 29, 1939.

and Rivers (5), other means were sought for the study of the relationship of these two antigens of vaccinia

The inhibition technique used in serological work assisted in establishing the existence of partially degraded forms of the A and B antigens of infectious myxomatosis of rabbits (6) These two protein-like antigens readily lose their specific precipitability on gentle heating or even on storage Solutions of A-antigen, which are no longer capable of precipitating with A-antibody, still combine with it as demonstrated by the inhibition technique Solutions of degraded B-antigen behave similarly Inhibition by the non-precipitable A- and B-antigens is specific, *i e*, each antibody combines only with the degraded form of its own antigen In view of the results obtained with myxoma antigens, inhibition of the L- and S-antibodies of vaccinia was investigated with the hope of demonstrating serologically degraded forms of the L- and S-antigens It was thought that experimentation with such degraded substances might provide data which would help in interpreting the interrelationship of the two native antigens The results of these investigations are reported in this paper

Materials and Methods

Source of Soluble Antigens—Dermal filtrate containing the soluble antigens of vaccinia was obtained from dermal pulp of rabbits which had been cutaneously infected 3 days previously with the C L strain of vaccine virus (1) The pulp from each rabbit was suspended in 30 to 40 cc of a 1 50 dilution of disodium phosphate citric acid buffer solution, pH 7 2 The suspension was freed of gross particles and of essentially all of the elementary bodies of vaccinia by differential centrifugation, and, finally, filtered through a Seitz pad to remove all residual virus These filtrates were stored at 3°C until used

Antisera—L-antiserum was prepared by absorbing S-antibody from hyperimmune antivaccinal serum Vaccine virus immune rabbits which had been given a course of intravenous injections of active elementary bodies of vaccinia provided the hyperimmune serum, absorption of S-antibody was carried out with proper amounts of heated dermal filtrate S-antisera were obtained from non-immune rabbits after several courses of injections of heat-inactivated elementary bodies

Precipitin Tests—0 25 cc volumes of serial dilutions of solution of antigen were prepared in physiological saline solution buffered at pH 7 2 and mixed with 0 25 cc amounts of an appropriate dilution of antiserum Readings were made after incubation overnight in closed tubes held at 50°C

EXPERIMENTAL

General Properties of L-Antigen—Only a few procedures have been found practicable for the concentration of L-antigen from filtrates of dermal pulp obtained from rabbits infected with the virus of vaccinia These methods are equally efficacious for the concentration of S-antigen Craigie and Wishart (1) have observed that both serologically active substances can be obtained

from virus-free dermal filtrate by bringing the pH of the solution to 4.5 and then redissolving the insoluble material thus obtained at pH 6 6 In our hands this method has proved the simplest and the most satisfactory for obtaining solutions containing large amounts of L- and S-antigens from which have been eliminated appreciable amounts of serologically inert material Moderate variations in the pH ranges employed for precipitating and for redissolving the antigens still result in final solutions with equal amounts of the two serologically active substances, in some instances, however, the recovery of antigenic material is less complete than it is with Craigie and Wishart's technique Bringing the pH to below 4 0 results in a loss of precipitability of L with its antibody, but S-activity is not appreciably reduced even at pH 1 8 On the alkaline side of neutrality both serological substances retain their activity for many days in the cold at pH 9 0 Furthermore, the L-antigen is unaffected by short exposure to borate buffer of pH 11 0, while the S-antigen is inactivated in N/20 NaOH only after minutes or hours depending on whether the procedure is carried out at 56°C or at 3°C Treatment with N/20 NaOH rapidly destroys the precipitability of L-antigen

Under the proper conditions, precipitation of concentrated unheated dermal filtrate with ammonium sulfate yields equal quantities of L- and S-antigen in the globulin fraction. L-activity is generally lost during fractional precipitation with ammonium sulfate unless filtrates which have been concentrated 10 to 20 times by evaporation are used as starting material, preliminary concentrations of dermal filtrate is unnecessary, however, if the objective is purification of S-antigen Evaporation of dermal filtrate through cellophane sausage casings suspended in an air stream at room temperature provides a ready means of concentrating the soluble antigens, but fails to eliminate any significant amount of serologically inert substances On the other hand, drying large volumes of dermal filtrate from the frozen state renders some of the inert material insoluble on subsequent resuspension. Although laborious, this procedure can be employed for concentration of the S-antigen, but it is often unsatisfactory for work with L-antigen since temporary thawing during the process may be followed by partial or complete loss of L-precipitability

The boiling of solutions containing antigens of vaccinia at pH values near neutrality with subsequent removal of coagulated protein has been found by several workers (5, 7, 8) to be of assistance in the partial purification of solutions of the heat-stable antigen, but obviously this procedure has no place in experiments designed for the purification of the heat labile material. Similarly, treatment of dermal filtrate with large volumes of cold alcohol results in complete disappearance of the substance capable of precipitating with L-antibody even though the stable antigen can be recovered from the alcohol insoluble fraction as has been demonstrated by others (5, 8)

The L-antigen of vaccinia may be regarded, therefore, as having certain

properties in common with S-antigen Both are insoluble in the neighborhood of pH 4 5 and soluble in the region of pH 6 5, and both are found in the globulin fraction when separated from concentrated dermal filtrate by partial saturation with ammonium sulfate The heat-labile antigen, however, readily loses its specific precipitability under conditions which do not alter the heat-stable antigen, *viz*, extremes of pH, treatment with alcohol, and, in certain instances, drying from the frozen state or precipitation with ammonium sulfate The common solubilities of the two antigens give no clue to their nature, since the data might equally well suggest that the antigens are parts of a single protein molecule, or that they are similar protein substances, or that L is a non-protein material associated with the S-protein molecule

Inhibition of L-Antibody

Solutions containing both L- and S-precipitable substances in high concentration showed no change in physical appearance when heated at 56°C for 1 hour This led us to think that the L-antigen, which was no longer demonstrable by the usual precipitation technique, might still be present in these heated solutions in an altered form which was unable to precipitate with L-antibody It seemed possible, furthermore, that such an altered antigen might inhibit L-antibody, and, indeed, this was found to be the case as shown by the following experiment

Experiment 1—1500 cc of dermal filtrate were placed in cellophane tubes and concentrated to 100 cc by evaporation The material was dialyzed against running water overnight, and while still in the original cellophane tube was again concentrated by evaporation to 50 cc This solution reacted in a dilution of 1 1024 with optimal amounts of both L- and S-antibodies Globulin material was obtained from the concentrate by fractional precipitation with ammonium sulfate 50 cc of a solution of the reprecipitated globulin material were treated with 5 cc of standard citric acid-disodium phosphate buffer, pH 4 5 Most of the material insoluble under these conditions redissolved when taken up in 20 cc. of diluted buffer solution, pH 6 6 This solution, containing globulin material insoluble at pH 4 5 and soluble at pH 6 6, had as much L- and S-activity as did the original concentrated dermal filtrate After heating at 56°C for 1 hour the solution no longer precipitated with L-antiserum but its titer with S-antiserum was not reduced One cc. of the heated solution was added to an equal amount of undiluted L-antiserum, the mixture was diluted with 4 cc of saline solution, incubated at 56°C for 1 hour, and stored at 3°C for 24 hours No precipitate appeared This treated mixture, which should have contained sufficient L-antibody to react strongly with unheated L-antigen, failed to form a precipitate when added to serial dilutions of unheated dermal filtrate and incubated under the usual conditions The inhibition experiment was repeated with identical results when portions of the same solution of antigen which had been heated at 60°C, 80°C, and 90°C for 1 hour were used

In the experiment just described L-antigen in the concentrated globulin material from dermal filtrate was affected in the usual manner by heating, *i.e.*, it no longer precipitated with L-antibody. A substance was present, however, in the heated solutions which was capable of inhibiting in some way the flocculation of L-antibody with unheated L-antigen. Since the partially purified material employed in the experiment was highly concentrated, it seemed desirable to repeat the inhibition procedure with a more dilute solution. For this purpose crude dermal filtrate prepared according to routine was used.

Experiment 2.—Dermal filtrate, pool 6, which precipitated in a dilution of 1:128 in the presence of optimal amounts of either L- or S-antibody was heated in a closed flask in a water bath at 80°C. for 1 hour. The heated filtrate which no longer precipitated with L-serum was added in varying amounts to 0.5 cc. portions of undiluted L-antiserum. Sufficient saline solution was added to each mixture to bring the final dilution of serum to 1:8 and the mixtures were incubated at 56°C. for 1 hour. Samples of the treated serum together with a control serum diluted only with saline, were tested for their capacity to precipitate with serial dilutions of unheated dermal filtrate, pool 6. The results summarized in Table I, show that the addition of an equal volume of heated filtrate to L-antiserum appreciably decreased the power of the serum to react with unheated filtrate. Furthermore, no precipitation occurred when the serum mixtures containing 3 and 5 volumes, respectively, of heat inactivated filtrate were tested against solutions known to possess active L-antigen.

TABLE I

Inhibition of L-Antibody by Heated Dermal Filtrate

Antigen	Treatment of L-antiserum 6314*	Dilution of antigen					
		1:8	1:16	1:32	1:64	1:128	1:256
Dermal filtrate 6 (untreated)	Control	+++	++++	++++	+++	+	−
	1 volume heated vaccinia dermal filtrate 6	++	++++	+++	±	−	−
	3 volumes heated vaccinia dermal filtrate 6	−	−	±	−	−	−
	5 volumes heated vaccinia dermal filtrate 6	−	−	−	−	−	−
	5 volumes heated myxoma dermal filtrate	++++	++++	++++	+++	±	−

* Dilution of antiserum was 1:8 in each test.

The data obtained in Experiments 1 and 2 indicate that crude or partially purified solutions containing either small or large amounts of L- and S-substances lose their capacity to precipitate with L-antibody after heating, but are, nevertheless, still able to inhibit L-antibody.

Inhibition of anti soluble substance antibodies of vaccinia has been ob-

served by others. Craigie and Wishart (1) found that certain heated crude dermal filtrates employed for absorption studies with antivaccinal serum contained inhibiting substances which were carried along with the absorbed serum and subsequently interfered with the demonstration of residual antibodies. These authors regarded the inhibitory substances as non-specific in character and found that fractionation of the filtrates resulted in their elimination. Salaman (9) likewise observed reductions in precipitin and agglutinin titers following absorption of antiserum with large amounts of dermal filtrate; he regarded these effects as examples of the familiar inhibition which occurs in the presence of an excess of antigen and thought that they should not be called non-specific. Since the L-antigen had been rendered non-precipitable by heating, the inhibition of L-antibody observed in Experiments 1 and 2 was certainly not dependent upon the presence of an excess of precipitable L-antigen. Moreover, that the inhibition was serologically specific was demonstrated in the following manner.

Experiment 3.—Dermal filtrate was prepared from rabbits cutaneously infected with the virus of myxomatosis by a technique essentially identical with that employed for rabbits infected with vaccinia. The filtrate which was rich in the A and B soluble antigens of myxoma was heated at 56°C. for 1 hour and then mixed with antivaccinal serum. The mixtures were tested for L-precipitins of vaccinia in the usual manner. As indicated by the results summarized in Table I, no inhibition of L-antibody occurred even when 5 volumes of the myxomatous filtrate were added to the L-antiserum. It has already been recorded that vaccine dermal filtrate fails to inhibit the anti soluble substance antibodies of myxomatosis (6).

Heated dermal filtrate appeared to be capable of specifically inhibiting L-antibody of vaccinia. It will be recalled that L-antisera are regularly prepared by absorbing hyperimmune antivaccinal serum with heated dermal filtrate; this absorption procedure when carried out under the proper conditions removes S-antibody and leaves L-antibody. This apparent contradiction was shown to depend on a quantitative factor, a much smaller amount of heated filtrate was generally needed to absorb S-antibody from immune serum than was necessary to obtain demonstrable inhibition of L-antibody.

Experiment 4.—Unabsorbed hyperimmune serum, number 6814, reacted in a dilution of 1:32 with an optimal amount of unheated filtrate, pool 6, and in a dilution of 1:4 with heated filtrate. Complete removal of S-antibody without a detectable reduction in the quantity of L-antibody resulted when the hyperimmune serum was absorbed with an equal volume of a 1:8 dilution of dermal filtrate, pool 6, which had been heated at 80°C. for 1 hour. L-precipitins, however, were no longer demonstrable in this antiserum after it was treated with an equal volume of four times concentrated solution of heated filtrate, pool 6. The filtrate had been concentrated by evaporation in a cellophane bag. Results of this experiment are summarized in Table II.

The material having the property of inhibiting L-antibody in the experiments so far presented always occurred in association with S-antigen It seemed desirable, therefore, to prepare purified S-antigen by the method of Parker and Rivers (5) and to test the power of this substance to block or inhibit L-antibody

Experiment 5 —625 cc. of dermal filtrate, pool 7, having a titer of 1 128 with optimal dilutions of L- and S-antisera were boiled and subsequently the globulin fraction was salted out with ammonium sulfate. The redissolved globulin fraction was precipitated with alcohol, redissolved in water, precipitated at pH 4 6 with citric acid-disodium phosphate buffer solution, and, finally, redissolved in dilute citric acid-disodium phosphate buffer solution, pH 8 0 This solution was boiled, the pH was then changed to

TABLE II

Preparation of L-Serum by Absorption of Immune Serum with Heated Dermal Filtrate and Inhibition of L-Antibody with the Same Material

Hyperimmune serum 6814	Antigen: Dermal filtrate 6 diluted 1:16	Dilution of serum						
		1 2	1 4	1 8	1:16	1:32	1 64	1:128
Untreated	Unheated	++++	++++	++++	++++	++++⁻	?	–
	Heated	++++	++	–	–	–	–	–
Absorbed*	Unheated	++++	++++	++++	++++	++++⁻	?	–
	Heated	–	–	–	–	–	–	–
Inhibited‡	Unheated		–	–	–	–	–	–

* Absorbed serum was prepared by treating 1.5 cc. of serum 6814 with 1.5 cc. of a 1 8 dilution of heated dermal filtrate 6.

‡ Inhibited serum was prepared by treating 6814 with an equal volume of 4 × concentrated solution of heated dermal filtrate 6.

6.0 by the addition of sodium hydroxide, the solution was again boiled and then dialyzed free of salts. 0.25 cc. of a 1 1024 dilution of this final solution gave a precipitate with optimal amounts of S-antibody A portion of the 41 cc. of final solution was dried from the frozen state and the residue was weighed, the solution before drying was shown to have contained 5 1 mg of dry material per cc. Hence, on the basis of precipitin titer and dry weight, it was estimated that 1 part in 800,000 was sufficient to give a specific reaction with S-antiserum The relative purity of the final preparation appeared to be of the same order as that obtained by Parker and Rivers. Portions of the final preparation of S-antigen as well as samples of different fractions obtained during the process of purification were tested in the usual way for their inhibitory effect on 0.5 cc. volumes of L-antiserum, number 6814 The results are summarized in Table III

The results summarized in Table III show that the substance in crude vaccine dermal filtrate capable of inhibiting L-antibody was also present in materials

obtained at intermediate stages and in the final stage of the purification of S-antigen These data indicate that L-inhibitor, like the precipitable form of L-antigen, is closely associated with S-antigen

Inhibition of S-Antibody

Earlier observations (10) indicated that S-antigen which has been heated with dilute alkali is not precipitated by its antibody The capacity of such a degraded antigen to combine with S-antibody was investigated by means of the inhibition technique The following protocol illustrates the results obtained in a typical experiment

Experiment 6—40 mg of the final dried preparation of S-antigen from Experiment 5 were dissolved in 8 cc. of N/20 NaOH The solution remained clear after heating at 56°C for 90 minutes A faint opalescence which appeared when the treated solution was brought to pH 7 2 with N/1 HCl was eliminated by ultracentrifugation at 30,000 R.P.M., the small amount of sediment thus obtained was discarded Serial dilutions of the clear supernatant fluid which contained practically all of the degraded S-antigen did not form precipitates when incubated with optimal amounts of S-antibody Varying quantities of the undiluted solution of treated antigen were mixed with 0 4 cc. volumes of S-antiserum, number 274, and with 0 3 cc. volumes of L-antiserum, number 6814, sufficient saline solution was added to each mixture to bring the final concentrations of antisera to their optimal dilutions The mixtures, all of which remained clear after incubation at 56°C for 1 hour, were tested for demonstrable precipitins against L- and S-antigens by the usual methods The results are summarized in Table IV

TABLE III

Inhibition of L-Antibody by a Preparation of S-Antigen

Antigen	Fraction prepared from heated dermal filtrate	L-antiserum treated with fraction	Dilution of untreated dermal filtrate				
			1 8	1 16	1 32	1 64	1 128
Dermal filtrate		None	+++	++++	++++	+++	+
	Globulin fraction	1 volume	–	–	–	–	–
	Alcohol insoluble fraction of globulin	1 "	–	–	–	–	–
	Final solution of S-antigen	1 "	++	++	+	–	–
	" " "	2 volumes	–	±	–	–	–

Dilution of L-antiserum was 1 6 in all tests. Inhibited sera were obtained by adding 1 or 2 volumes of test material to 0 5 cc. amounts of L-antiserum S-antigen titer of each test material was 1 1024 Final solution of S contained 5 mg per cc

The data presented in Table IV indicate that S-antigen of vaccinia which has been degraded by heat and alkali to a stage where it no longer precipitates with

its antibody is still able to combine with this antibody For example, 0.5 mg of treated antigen completely inhibited the antibody in 0 4 cc. of undiluted S-antiserum It is also evident that this preparation of degraded antigen had only a slight capacity to inhibit L-antibody That the property of the degraded S-antigen to inhibit L-antibody was indeed less than that of the purified precipitable S-antigen from which it was derived may be seen by comparing the data given in the protocols of Experiments 5 and 6, for instance, 2 to 3 times as much of the alkali treated material was needed to produce the slight inhibitory effect on L-antibody observed in Experiment 5 where undegraded S-antigen was used

TABLE IV

Inhibition of S-Antibody

Antigen	Antiserum	Treatment of antiserum with degraded S	Dilution of antigen				
			1 8	1:16	1:32	1 64	1 128
		mg					
Dermal fil trate	S (diluted 1 4)	None	+++-	++++	++++	++++	++
		0 125	+++	++++	++++	++++	++
		0 25	?	+++	++++	+	–
		0 50	–	–	–	–	–
		0 75	–	–	–	–	–
		1 00	–	–	–	–	–
	L (diluted 1 6)	None	+++	++++	++++	++++	++
		1 00	++++	++++	++++	+++	+
		7 50	+++	++	++	–	–

0 4 cc. volumes of S-antiserum and 0.3 cc. volumes of L-antiserum were treated with the designated amount of S-antigen which had been heated at 56 C for 90 minutes in the presence of N/20 NaOH.

Inhibition of S-antibody can also be demonstrated by adding the antibody directly to mixtures of native and degraded S-antigen In this type of experiment, however, the latter material must be present in large amounts in comparison to the former This is illustrated by the data presented in Table V which summarizes the results obtained when alkali and heat treated S-antigen from Experiment 6 was added in increasing amounts to 0 4 cc. volumes of unheated dermal filtrate, after which serial dilutions of the mixtures were prepared and incubated with optimal amounts of L- or S-antibody Results of the titrations made with the mixture containing 0 1 mg of degraded S-antigen were comparable to the controls. The presence of 1 0 mg of the non-precipitable or degraded antigen prevented flocculation of the native S-antigen only in the lowest dilutions of the titrations with S-antibody, while 5 0 mg completely blocked precipitation of native S-antigen with its antibody through

out the range of dilutions In none of the mixtures was the reaction with L-antibody appreciably altered

On several occasions S-inhibitor has been encountered in preparations of vaccine dermal filtrate that were not subjected to alkaline treatment. In these experiments the filtrates were concentrated 10 to 20 times before boiling The technique for purifying S-antigen was originally applied to crude dermal filtrates (5) In order to avoid working with large volumes, we first concentrated the crude filtrates by evaporation in cellophane tubes The results obtained when the purification procedure was applied to these concentrates were disappointing in that yield of S-antigen was negligible as determined by pre-

TABLE V

Inhibition of S-Antibody—Continued

Antigen mixture		Antiserum	Dilution of original dermal filtrate			
Dermal filtrate	Degraded S-antigen		1 8	1 16	1 32	1 64
cc	*mg*					
0 4	0 1	L	++++	++++	++++	++
		S	++++	++++	++++	+++
0 4	1 0	L	++++	++++	+++	++
		S	–	++++	++++	+++
0 4	2 0	L	++++	++++	+++	++
		S	–	–	–	++
0 4	5 0	L	++++	++++	+++	+
		S	–	–	–	–

Mixtures of dermal filtrate and degraded S-antigen were incubated at 37°C for ½ hour and then diluted serially Constant amounts of L-antiserum 6814 diluted 1 6 and of S-anti serum 274 diluted 1 4 were added and the titration results were read after incubation over night at 50°C

cipitin tests The failure to demonstrate the expected amounts of S-antigen in the solutions was found to be dependent upon the presence of an inhibitor which could be removed, leaving behind precipitable S-antigen The results of this type of work are illustrated by the following experiment

Experiment 7 —960 cc. of dermal filtrate, pool 8, were dialyzed in cellophane tubes against running water and then concentrated to 90 cc. in the same tubes by evaporation in a stream of air The clear solution became opalescent after gentle boiling for 5 minutes, and the small amount of precipitate that formed was removed by centrifugation The globulin fraction obtained by precipitation with ammonium sulfate was redissolved in water, dialyzed free of sulfate ions, and brought back to a volume of 90 cc. The original crude dermal filtrate in a dilution of 1 64 precipitated with L- and S-antisera, but the concentrated solution of heated globulin gave only a slight precipitate in dilutions of 1 8 to 1 32 with S-antiserum A portion of the solution

of globulin was fractionated by the method of Craigie and Wishart (1) for the con centration of LS-antigen Only about half of the voluminous white precipitate obtained at pH 4 5 redissolved when it was suspended in buffer solution, pH 6 6 The solution containing the material which dissolved at pH 6 6 had a precipitin titer of 1 128 when tested with S-antiserum The material which failed to dissolve at pH 6 6 was soluble in dilute buffer solution pH 9 0, and the solution remained clear when brought back to pH 7 0 While this solution did not precipitate in dilutions of 1 4 to 1 512 in the presence of an optimal amount of S-antibody, it was found to have a marked inhibiting effect on S-antibody S-antiserum treated with 3 volumes of the solution would not precipitate with native S-antigen On the other hand, L-antiserum was not appreciably inhibited by 5 volumes of the solution.

The results obtained in Experiment 7 throw some light on the work of Craigie and Wishart (1) For example, it was shown that under certain conditions the heating of dermal filtrate, even in the pH range near neutral reactions, may result in the formation of S-inhibitor Furthermore, it was found that S-inhibitor can be separated from precipitable S-antigen by means of the different solubilities of the two substances at several pH values

DISCUSSION

The results of the foregoing experiments may be briefly summarized as follows Dermal filtrate prepared from the skin of rabbits infected with the virus of vaccinia contains the heat labile (L) and heat-stable (S) antigens of vaccinia which can be demonstrated by the precipitation technique. Gentle heating destroys the precipitability of L-antigen but leaves in solution a substance capable of inhibiting L-antibody This L-inhibitor, like precipitable L-antigen, is closely associated with S-antigen and cannot readily be separated from it. Preparations of S-antigen can also be degraded by any of several methods to a stage where precipitation with S-antibody does not occur, how ever, inhibition of S-antibody with this material is easily demonstrated De graded S-antigen in contrast to precipitable S-antigen, has little power to inhibit L-antibody The solubilities of S-inhibitor are different from those of S-antigen and L-inhibitor

The immediate objective of the present experiments, *viz*, the demonstration of degraded forms of L- and S-antigens of vaccinia which are capable of in hibiting their specific antibodies, was accomplished The studies were less successful, however, in regard to their ultimate objective which was the elucida tion of the relationship between L- and S-antigens Nevertheless, the observations just reported may serve as the basis for several hypotheses on the relationship of these two serologically active substances

One of the simplest of the hypotheses is as follows The native antigen present in infected tissue is L, or the antecedent substance from which it is derived. L is readily degraded during manipulation and storage to a slightly modified

material, S, which is still capable of eliciting a specific antibody in animals and which combines with L-antibody as demonstrated by inhibition tests S-antigen can be further degraded to a stage where it inhibits but fails to precipitate with its antibody, and, in this form it loses most of its ability to combine with L-antibody A graphic representation of the various stages of degradation according to this scheme is presented in Text-fig 1 There are

Hypothesis	Native materials in dermal filtrate	Effect of heat	Effect of heat and alkali
I	L Precipitates with L-antibody	S Inhibits L-antibody Precipitates with S-antibody	S' No inhibition of L-antibody No precipitation with S antibody Inhibits S-antibody
II	L Precipitates with L-antibody S Precipitates with S-antibody	L' Inhibits L-antibody S Precipitates with S-antibody	L" No inhibition of L-antibody S' Inhibits S-antibody
III	(L S) Precipitates with L-antibody Precipitates with S antibody	(L' S) Inhibits L-antibody Precipitates with S antibody	(L" S') No inhibition of L-antibody No precipitation with S antibody Inhibition of S-antibody
	Other theoretical combinations		
	(a) (L S') Precipitates with neither antibody Inhibits both antibodies	(b) (L" S") Neither precipitates nor inhibits	(c) (L S') Precipitates with L-antibody No precipitation with S antibody Inhibits S-antibody

TEXT-FIG 1

several serious objections to this hypothesis No reasonable explanation is at hand for the regular occurrence of equal amounts of L- and S-antigen in preparations from infected tissue Moreover, it should be possible, if the hypothesis were correct, to demonstrate a simultaneous increase in S along with a decrease in L during the various procedures which render L-antigen non-precipitable

A second hypothesis might begin with the assumption that L- and S-antigens are separate protein molecules which are distinguishable by serological means but not by ordinary physical and chemical procedures According to this idea each of the antigens could be degraded independently, but, since L is more

easily affected than S, the labile antigen would generally be denatured one step further than the stable one (Text fig 1) The similar physicochemical nature of the native substances might also be considered to be characteristic of the mildly degraded antigens, thus, a separation of L-inhibitor and S-antigen would be difficult This hypothesis, like the first, affords no explanation for the uniform ratio of L- and S-antigens in crude filtrates Furthermore, it is hard to reconcile it with the almost invariable results obtained in absorption experiments, namely, the removal of both antigens on the addition of either antibody

A third hypothesis deals with an LS-complex similar to that postulated by Craigie and Wishart (1) No assumptions need be made about the chemical nature of the L-part of this complex or about the type of union between it and the protein like S-part. As in the second hypothesis, the L-reacting substance would be considered to be degraded more readily than the S under ordinary conditions, and complexes might be formed of L′S and L″S′ on treatment with heat or heat and alkali, respectively (Text fig 1) In such a scheme the first degradation product, *e g*, L′ would inhibit L-antibody but not be precipitated by it, while L″ would have lost most of its capacity to inhibit L-antibody Similarly, stages in the degradation of the S-portion of the complex could be postulated Additional complexes with various combinations of native and degraded stages of the L- and S-parts might be considered, several of these are represented in Text fig 1, (*a*), (*b*), (*c*) The objections raised to the first two hypotheses are not valid for the last one The unusual results of occasional absorption experiments reported by Parker (2) and Craigie and Wishart (3) can be explained by this hypothesis if one assumes that under certain rare conditions a portion of the LS-complex in dermal filtrate is degraded to a form represented as LS′ (Text fig 1, (*c*)) A mixture of this complex and native LS when treated with S-antibody could result in the precipitation of LS, leaving in solution LS′ combined with S-antibody Such a soluble antigen-antibody combination might then be precipitated on the addition of L-antibody

At the time these experiments were completed we were inclined to accept the third hypothesis as the one which more nearly explained the available date on the relationship of the L- and S-antigens of vaccinia. Work reported in accompanying and subsequent papers (4, 11) has done much to strengthen our belief that this hypothesis is substantially correct.

CONCLUSION

Experimental data are presented which may be interpreted as follows. The heat labile (L) and heat-stable (S) antigens of vaccinia occur in nature as a complex consisting of a single substance with two serologically active parts, each of which may be degraded independently of the other

We wish to express our appreciation to Dr Kenneth Goodner for his advice

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THE LS-ANTIGEN OF VACCINIA

II Isolation of a Single Substance Containing Both L- and S-Activity

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The intimate association of the heat labile (L) and heat-stable (S) soluble antigens of vaccinia was shown by the work of Craigie and Wishart (1) Moreover, observations on the degraded forms of these antigens, reported in the previous paper (2), further indicated the close relationship of these two antigens The fact that the L-antigen is readily denatured by a number of procedures which are ordinarily used for purification of immunologically active substances has delayed the acquisition of knowledge concerning its nature. On the other hand, the heat stable antigen has been studied in considerable detail (3, 4) and has been shown to be a protein substance Although the results of several experiments (4, 5) have been interpreted as indicating that L-antigen may be obtained free of the S-antigen, these experiments are also subject to a different interpretation (2) The work reported here was undertaken in the hope of obtaining pure L-antigen in sufficient amounts to study its physical and immunochemical properties As will be shown, the L-antigen is not found free from the S-reacting substance under ordinary conditions Indeed, the results indicate that the L- and S-immunological activities reside in a single native protein molecule A method for isolating pure LS-antigen of vaccinia from virus-free filtrates of infected rabbit skin will be given in this communication together with observations on the physical and immunological properties of the native substance and of some of its degraded forms

Materials and Methods

Vaccine Dermal Filtrate—Dermal filtrate was available in large quantities as a by-product obtained from the preparation of washed elementary bodies of vaccinia which were used for other studies The technique of Craigie (6) was employed without significant modification Dermal pulp from each cutaneously infected rabbit was suspended in 30 cc. of a 1 50 dilution of standard phosphate buffer solution pH 7 2 and the solution which remained after removal of tissue particles and elementary bodies by means of differential centrifugation was filtered immediately through a Seitz pad. Bacteriologically sterile virus-free filtrates were stored at 3°C. until 800 to 1200 cc. had been accumulated usually a period of a month

Antiserum—L-antiserum used throughout this portion of the work was prepared from pooled sera obtained by bleeding vaccine virus immune rabbits which had been hyperimmunized with washed active elementary bodies of vaccinia, the serum was absorbed free of S-antibody with heated crude dermal filtrate S-antisera were obtained from rabbits which had been immunized with a non-infectious solution of purified S-antigen (4) Precautions were taken to prevent these non immune animals from becoming accidentally infected with vaccinia during the period of immunization Our experience, like that of Parker, indicated that solutions of S antigen prepared in this manner elicit a poor antibody response in rabbits and in order to obtain potent sera several courses of treatment were given For the final course of immunization, washed and graded collodion particles were added to the solution of S-antigen and the mixture was injected intravenously

Serological Methods—The serological techniques employed in this work have been reviewed in a previous paper (2)

Electrophoretic Technique—Electrophoretic experiments were carried out in the Tiselius apparatus with the schlieren-scanning optical system (7) the details of which have been discussed elsewhere The temperature of the thermostat containing the cell was regulated at 1°C, but all the values of mobility given in this paper have been corrected to 0°C

EXPERIMENTAL

Observations on Concentrated Whole Dermal Filtrate—The results obtained in studies in which the electrophoretic technique was applied to concentrated dermal filtrate, both before and after heating, are illustrated in the following experiments

Experiment 1—One liter of dermal filtrate was concentrated to a volume of 100 cc by means of evaporation through a cellophane sausage casing placed in an air stream at room temperature for 24 hours The L- and S-titers of this concentrate were 1 1000 A portion of the material was dialyzed against 0 05 M lithium-veronal buffer solution at pH 7 9[1] Upon electrophoresis this dialyzed material was found to display four boundaries, all of which corresponded to negatively charged substances at this value of pH A reproduction of the electrophoretic pattern obtained is shown in Fig 1 The mobilities of the four components, which were designated I to IV, were $6\ 7 \times 10^{-5}$, $5\ 4 \times 10^{-5}$, $4\ 4 \times 10^{-5}$, and $3\ 4 \times 10^{-5}$ cm /sec per volt/cm, respectively In Fig 1, as in subsequent figures, the first peak on the right, ϵ, does not correspond to any component, but is due to a gradient of buffer salts

Experiment 2—A portion of the same material was heated at 70°C for ½ hour in a closed vessel (a procedure which inactivates L without destroying S) and an electro

[1] This solution contained diethylbarbituric acid, its lithium salt, and lithium chloride, each at 0 025 M The chloride was used to build up the ionic strength, since the diethylbarbituric acid concentration is fixed by its relatively low solubility at 0°C

phoretic determination was made under conditions identical with those described above. The electrophoretic pattern obtained in this experiment is illustrated in Fig 2.

It is immediately obvious from Figs 1 and 2, that the results obtained in the two experiments were strikingly different. As a result of heating, one component (III) had completely disappeared and another component (II) had been

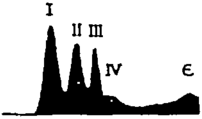

Fig 1

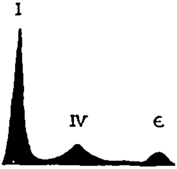

Fig 3

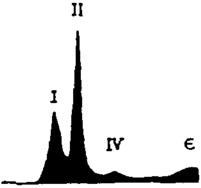

Fig. 2

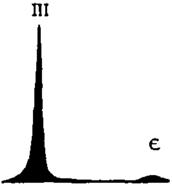

Fig 4

Fig 5

increased in amount, on the other hand, components I and IV were unaffected by heating under these conditions. A quantitative estimation of the relative amounts of components II and III in these two preparations was obtained from the measurement of the areas in the electrophoretic patterns This indicated that the increase in component II which occurred on heating was approximately equal to the loss of component III

When the observations described above were first made we were not certain whether L- and S-activities were associated with one or with two substances

Therefore, we were inclined to believe from these data that component corresponded to L-antigen and that component II corresponded to S-anti furthermore, that as a result of heating, L had been transformed to S L be said at once that this hypothesis was subsequently proved wrong Ne theless, we immediately attempted to isolate by electrophoretic separation four components shown in Fig 1, in order to identify the substances sero cally These results may be briefly summarized as follows components I, fastest, and IV, the slowest, were readily isolated and were found to be s logically inert Sharp electrophoretic separation of components II and III not possible, due to the fact that their respective mobilities were not s ciently different for such resolution Even when electrophoresis was car out in solutions with pH values from 6 0 to 8 6, such fractions which v obtained invariably showed L- and S-activity in equal amounts Since above methods were inadequate for the purpose, fractionation of the L- S-antigens from crude dermal filtrate was attempted by other means

Fractionation of Dermal Filtrate by Precipitation—It has been shown Craigie and Wishart (1) that the L- and S-antigens can be concentrated fi crude dermal filtrate by precipitating at pH 4 5 and redissolving the precipit at pH 6.5 This procedure enabled these workers to eliminate a considera amount of serologically inert nitrogenous material, present in the crude filtra without appreciably reducing the titer of the antigens in the final preparati Parker and Rivers (3), in their experiments dealing with the purification S-antigen from heated dermal filtrate, had also observed that the heat-sta active substance has similar solubility characteristics Procedures based these observations were employed in the fractionation of concentrated derr filtrate and the fractions thus obtained were studied electrophoretically a serologically The following experiment illustrates the methods employ and the results obtained

Experiment 3—1150 cc. of vaccine dermal filtrate, collected over a period of 5 wee were concentrated by evaporation from cellophane bags to a volume of 110 cc T slight amount of insoluble material present in the concentrate was removed by c trifugation and discarded The clear concentrate was again placed in a celloph sack and dialyzed overnight against 4 liters of unbuffered physiological saline soluti at 3°C The sack and its contents were then suspended in 450 cc of 0 05 M aceta buffer solution at pH 4 63, also at 3°C Precipitation began at once at the surf of the cellophane membrane The sack was agitated from time to time and by t end of 3 hours no further increase in the precipitate could be noted The mater was then removed from the sack and the precipitate was separated by centrifugati in the cold The clear, straw-colored supernatant fluid, which had a pH of appro mately 4 6, was set aside for further study, this was designated fraction A T oyster-gray sediment, which occupied a volume of about 2 cc in the centrifuge tul was washed in the cold with 45 cc. of the same acetate buffer solution The rese

mented, washed material was then taken up in 50 cc. of 0 05 M cacodylate buffer solution at pH 6.31 and dialyzed overnight against 450 cc. of the same solution at 3°C. Approximately half of the material dissolved under these conditions The material insoluble at pH 6 31 and the substance dissolved at this pH were separated by centrifugation The clear, pale straw-colored supernatant solution was labeled fraction B and stored The sediment was washed with the cacodylate buffer solution and then suspended in 50 cc. 0 05 M veronal buffer solution of pH 8 56 Following dialysis against a large volume of the same buffer solution an appreciable part, but not all of the material dissolved The clear liquid was again separated from the precipitate by centrifugation and designated fraction C. The precipitate which was insoluble at pH 8 56 dissolved partially in 0 1 M borate buffer solution pH 11 0 and almost completely in 0 05 M NaOH, since all the material in dermal filtrate was originally soluble in neutral solutions this portion probably consisted largely of denatured products.

The three fractions which had been saved for further study were A the material which remained soluble at pH 4 63 B the material which was insoluble at pH 4 63 but soluble at pH 6.31 and C, the material which was insoluble at pH 4 63 and 6.31, but soluble at pH 8 56 Portions of fractions A and B were placed in cellophane sacks and dialyzed against 0 05 M lithium veronal buffer solution at pH 7 91 for 1 or 2 days in the cold, after which they were examined by electrophoresis similar data were obtained on fraction C in veronal buffer solution pH 8 56 The serological properties of all three fractions were investigated

Fraction A was found to contain only two components, these had the same electrophoretic mobilities as components I and IV in whole dermal filtrate Fraction B consisted of a single substance which was electrically homogeneous and which moved at a rate corresponding to that of component III Fraction C contained a trace of component III, but most of the material appeared to correspond to component II in whole filtrate The electrophoretic patterns obtained with fractions A, B, and C are shown in Figs 3, 4, and 5, respectively

The results of serological studies on the original material and on fractions A, B, and C are summarized in Table I Briefly it may be stated that fraction B, consisting of the electrically homogeneous component III, contained practically all of the L- and S-reacting material present in the original concentrated dermal filtrate It gave a precipitate when diluted 1 1600 and incubated in the presence of an optimum amount of L- or of S-antibody The small amount of L- and S-precipitinogen activity found in the C fraction was completely lost after refractionation, furthermore all of the serologically active material was now recovered in the solution corresponding to the original B fraction The A fraction was serologically inert.[2]

This portion of the work may be summarized as follows. Components I and

[2] In several early experiments in which dialysis of crude filtrate against pH 4 6 buffer solution was inadequate appreciable amounts of components II and III were left in fraction A.

IV found in vaccine dermal filtrate may be separated quantitatively from components II and III by precipitation of the latter at pH 4 6 Furthermore, component III can be isolated from this precipitate in uncontaminated form by extracting this precipitate at pH 6 3 Such a solution of component III contains practically all of the L- and S-reactive substance present in the original filtrate

Observations Indicating that a Single Substance in Its Native State Contains Both L- and S-Activity—In our laboratory, precipitation titrations with the

TABLE I

Serological Activity of Fractions Derived from Vaccine Dermal Filtrate

Test solution	Antiserum	Dilution of test solution							
		1 25	1 50	1 100	1 200	1 400	1 800	1 1600	1 3200
Concentrated dermal filtrate	L		++++	++++	++++	++++	+++	++	+
	S		±	+++	++++	++++	++++	+++	+
Fraction A	L	−	−	−	−	−			
	S	−	−	−	−	−			
Fraction B	L		++++	++++	++++	++++	+++	++	±
	S		±	++++	++++	++++	++++	++	±
Fraction C	L	++++	++++	+++	+	±			
	S	++++	++++	+++	++	+			
Refractionated C	L	±	−	−	−	−			
	S	+	±	−	−	−			

L-antiserum 1601 was used in a dilution of 1 2 throughout. S-antiserum 493 was used in a dilution of 1 8 0 01 M lithium-veronal buffer solution, pH 8 6, containing 0 9 per cent of NaCl served as diluent. Test mixtures were incubated at 50°C overnight.

See text for preparation of test materials

soluble antigens of vaccinia are ordinarily carried out by incubating the antigen-antibody mixtures at 50°C for 18 hours in closed tubes Such an incubation period is sufficient to degrade the heat-labile antigen, L, to a state where it no longer precipitates with its antibody Consequently, at this stage in the experiments it was necessary to consider the possibility that the material designated as component III was a substance which possessed only L-activity in its native form According to such an hypothesis, this native material could be degraded by heating to a substance with the electrical mobility of component II and with S-activity

In order to test this hypothesis a solution containing *component III* was diluted serially in the usual manner, and portions of each dilution were placed in tubes with

optimal amounts of L- or of S-antibody Four sets of duplicate titrations were thus made and each set was incubated for 18 hours at a different temperature namely, at 3°, 20°, 37°, and 50°C.

In each set of titrations the precipitin endpoint in the presence of S-antibody was identical with that obtained in the presence of L-antibody Flocculation was slower at the lower temperatures, and with incubation at 3 C. periods of 48 to 72 hours were necessary in order to develop maximum precipitation end-points thus obtained equalled those observed after 18 hours at 50°C.

The presence of an S-reactive substance in solutions of component III which were incubated at temperatures that do not change the precipitability of the heat labile substance, clearly shows that component III in its native state possesses both L- and S-reacting portions, therefore, S cannot be a degradation product of L-antigen Observations reported in succeeding sections will clarify the relationship between component II and heated component III.

Evidence based on absorption experiments in which solutions of component III were used with L- and with S-antisera indicates that the two antigenic parts of component III cannot be separated by this method The following protocol illustrates the results obtained with one of several preparations of antigen absorbed with antisera

Experiment 4 —40 cc. of an electrophoretically homogeneous solution of component III were obtained from 800 cc. of vaccine dermal filtrate by the method described in the protocol of Experiment 3 The solution had a titer of 1 2048 with L- and with S-antibody A 0 7 cc. volume of S-antiserum 493 was mixed with 0 1 cc. of the solution of antigen in one instance, and in another with 0 05 cc. of antigen and 0 05 cc. of saline. Each antigen antibody mixture was diluted with an equal volume of saline solution, and incubated at 37°C for 4 hours and then at room temperature for a similar period of time The voluminous precipitate which formed was removed by centrifugation Similar absorptions were carried out with 0 8 cc. volumes of L-anti serum 1601 and varying amounts (0 01 to 0 10 cc.) of solution of antigen, sufficient saline solution being added to each mixture to bring the final volume to 1 6 cc. These were tested for residual antigen and for residual antibody The results of these tests which are summarized in Table II show that absorption with either L- or S-antibody removes both L- and S-antigenic substances completely or reduces both to the same degree.

It is apparent from these observations that the electrically homogeneous[3] component III, which was obtained from the dermal filtrate, contains both L- and S-activity and that the two serologically distinct parts are inseparable in the native substance. Ultracentrifugal and chemical studies on this material, which will be reported in another paper, showed it to be a homogeneous protein

[3] Studies on the material at values of pH between 6.2 and 8 6 consistently revealed but one electrophoretic component These measurements are discussed in detail in a subsequent paper

TABLE II

Absorption of Solutions of Component III with L- and S Antibody

Absorption mixtures			Residual antigen									Residual antibody			
			Dilution of original antigen solution									Dilution of absorbed mixture			
Antigen	Antiserum type	Anti sera	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	1 2048	1 4096	1 1	1 2	1 4	1 8
cc	*cc*														
	Unabsorbed	L		++++	++++	++++	++++	+++	++	+	−				
		S		−	±	++++	++++	+++	+++	+	−				
0 01	Anti S 0 7	L	−	−	−	−	−								
		S	−	−	−	−	−					−	−	−	−
0 05	Anti S 0 7	L		−	−	−	−	−							
		S		−	−	−	−	−				+++	++++	−	−
0 1	Anti L 0 8	L		++++	++++	+++	+	±				−	−	−	−
		S		++++	++++	+++	++	±							
0 05	Anti L 0 8	L		++	±	−	−	−				−	−	−	−
		S		++	±	−	−	−							
0 025	Anti L 0 8	L			−	−	−	−				++	−	−	−
		S			−	−	−	−							

L-antiserum 1601 was prepared from pooled sera of rabbits hyperimmunized with active elementary bodies of vaccinia by absorption of S-antibody with heated dermal filtrate S-antiserum 493 was obtained from a rabbit immunized with a non infectious fraction of heated dermal filtrate rich in the heat stable soluble antigen of vaccinia. Tests for residual antigen were carried out with optimal dilutions of antisera, *i e*, 1 2 for L- and 1 8 for S-antisera Determinations of residual antibody were made by adding 0 25 cc of a 1 16 dilution of unheated vaccine dermal filtrate to equal volumes of serial dilutions of the absorbed mixture

See text for details of absorption technique

with a molecular weight near that of serum globulins On the basis of electrophoresis and ultracentrifugal evidence, therefore, we conclude that component III is a single molecular substance containing both L- and S-activity Henceforth we shall designate it as LS-antigen This LS-antigen is probably the substance that Craigie and Wishart (1) prepared from the same type of source material and called "LS fraction" Our method of isolation of the antigen is similar to theirs and the serological data obtained following incubation at various temperatures and by absorption with L- and S-antibody agree with their findings The present experiments supplement the observations of these authors in that they were done with solutions of antigen which were proved to be pure by a number of different criteria and hence these experiments carry additional weight in theoretical discussions of the nature of LS-antigen

Effect of Heat on LS Antigen—Destruction of the precipitability of the labile soluble substance of vaccinia by heating has been well established (1, 5) The combination between heated L- antigen and L-antibody without subsequent flocculation has been discussed in the preceding paper (2) Since pure LS-antigen was now available it seemed desirable to study the effect of heat on its physical and serological characteristics

Experiment 5—The observations described in the following experiment are typical of those noted in the study of several different preparations of LS-antigen A portion of the solution of LS-antigen from Experiment 4 was heated for ½ hour in a water bath at 70°C. in a closed tube. The solution which had been clear now showed a faint opacity After removing the trace of insoluble material by high speed centrifugation (12,000 R.P.M.) the solution was redialyzed against 0 05 M lithium veronal buffer solution at pH 7 91 and was examined by electrophoresis

The electrophoretic mobility of this lot of unheated LS-antigen under these conditions was $4\ 0 \times 10^{-5}$ cm /sec. per volt/cm After heating this solution, a single electrically homogeneous component was still present, but it now moved at a rate of $5\ 9 \times 10^{-5}$ cm /sec. per volt/cm This preparation of heated LS was inhomogeneous when examined in the analytical centrifuge as will be shown in another paper in this series. The precipitin titer of the solution of unheated LS was 1 2048 with both L- and S-antibody the heated LS still titered 1 2048 with S-antibody but failed to precipitate over the range of dilutions from 1 8 to 1 4096 in the presence of L-antibody

This experiment throws considerable light on the early observations which misled us into believing that components II and III represented S- and L-antigens respectively Heating destroyed the precipitability of the L-part of the LS-antigen and increased the mobility of the degraded molecule to a value almost identical with that of component II in crude dermal filtrate The augmentation of component II concurrent with the disappearance of component III in heated crude filtrate thus appears to have been a coincidence

The Occasional Occurrence of Partially Degraded LS-Antigen in Crude Dermal Filtrate—Although pure LS-antigen had been previously obtained in four successive experiments by means of the fractionation technique which has been described, it was found in one experiment that the fraction which usually contained only LS-antigen was contaminated with a component having the mobility of either component II or of heated LS-antigen

Experiment 6—850 cc of dermal filtrate were concentrated and fractionated in the manner described in Experiment 3 Fraction B was examined by electrophoresis in 0 05 M lithium-veronal buffer pH 7 89 and found to contain two components, one component corresponding to LS-antigen was present in an appreciable amount, and the other, corresponding to either heated LS-antigen or component II, was estimated to be present in about ½ the concentration of LS-antigen Although the titer of the solution was 1 1600 with L-antibody and 1 3200 with S-antibody this was considered inconsequential at the time Accordingly, the solution was refractionated to remove the contaminating material In short, the material was precipitated three times at pH 4 6 and each time the precipitate redissolved almost completely at pH 6 6 The final preparation gave essentially the same electrophoretic pattern and serological titers as did the original fraction B

Refractionation of the solution which might have been expected to eliminate the contaminating material if it were component II failed to accomplish this Furthermore, since the contaminant had solubility characteristics similar to those of LS-antigen it appeared likely that it was a degraded form of LS-antigen Therefore, at this point, it seemed desirable to know whether the technique of fractionation, as regularly employed, was adequate to separate heat-degraded LS-antigen from native LS-antigen

Experiment 6 (Continued)—14 cc of the three times fractionated solution B from Experiment 6 were still available The solution had an S-titer of 1 1600 and L-titer of 1 800 One half of the material was heated at 70°C for ½ hour This portion then failed to react with L-antibody but still retained its S titer of 1 1600 The two solutions were recombined, the precipitin titers were then 1 400 for L-antigen and 1 1600 for S-antigen, as might have been expected

After dialysis, the mixture was again studied electrophoretically, the pattern revealed the presence of two components with mobilities corresponding to LS-antigen and heated LS-antigen, which were present in concentrations closely corresponding to their respective titers The material was then subjected to the solubility fractionation process and was again examined electrophoretically No change in the resulting pattern was noted The serological titers remained the same

Essentially similar electrophoretic and serological observations were made on a mixture containing equal amounts of solutions of unheated and heated LS antigen from Experiment 3 In this case, however, the two electrical components were about equal quantitatively, and the solution had titers of 1 800 to 1 1600 with L- and S-antibody respectively

The results obtained in Experiment 6 indicate that heat-degraded LS-antigen has essentially the same solubility characteristic as native LS-antigen and hence cannot be separated from it by the procedures employed This is of importance for it indicates that, in general, dermal filtrate contains no partially degraded LS-substance, *i.e*, one possessing S-activity but not L-activity In other words, all of the L- and S-activity in fresh dermal filtrate is carried by component III In the single experiment in which pure LS-antigen was not isolated by the process of fractionation it appears that the contaminant was a degraded form of antigen similar to heated LS-antigen. The reason for the appearance of this contaminant in a single pool of dermal filtrate is not at hand It may be pointed out, however, that, unlike the usual preparations, in this case, several of the lots of the angle supernatant fluid from the suspension of crude pulp had not been filtered immediately, but were allowed to stand at ice box temperature for several days before being passed through a Seitz pad The centrifuged but unfiltered fluid is slightly opalescent and contains some virus, tissue debris and bacteria, and the presence of one or more of these might well have contributed to partial denaturation of LS-antigen by enzymatic digestion or otherwise

Inhibition of L- and S Antibodies by Degraded Forms of LS Antigen—The interpretation of the experiments described in the previous paper (those dealing with inhibition of anti-soluble-substance antibodies of vaccinia by various preparations of materials from vaccine dermal filtrate) was difficult, due to the lack of critical evidence showing that the degradation products of a single substance were capable of inhibiting the two antibodies Accordingly, solutions of pure LS-antigen were treated with heat alone, and also with heat and dilute alkali, and the specific inhibitory properties of the resulting preparations were studied (Experiments 7 and 8)

Experiment 7—A portion of solution obtained from Experiment 3 was heated at 70°C. for ½ hour The resulting solution still precipitated in a dilution of 1 1600 with optimal amounts of S-antibody but failed to flocculate when dilutions of 1 25 to 1 3200 of the solution were mixed with optimal amounts of L-antibody Inhibition tests were made in the following manner Varying amounts of two different L-antisera were treated with varying amounts of heated LS-antigen solution Sufficient saline solution was added to the test mixtures to make the final dilutions of serum comparable. The mixtures were incubated at 50°C. for ½ hour and then at 37°C. for an additional ½ hour The solutions remained clear and centrifugation failed to sediment an appreciable amount of material. The test sera, together with controls, prepared by properly diluting antisera with saline and incubating for a similar period, were then set up in constant amounts with serial twofold dilutions of unheated crude dermal filtrate Illustrative data obtained are summarized in Table III.

It is evident from the results summarized in Table III that a solution of LS-antigen, shown to be pure by electrophoresis and ultracentrifugation, when

degraded by heat to the stage where it fails to give a precipitin reaction with L-antibody, is still capable of combining with L-antibody as demonstrated by the inhibition technique An additional point of interest may be mentioned regarding the serological behavior of LS-antigen degraded by heat Mixtures of serial dilutions of such antigen and optimal amounts of L-antibody, which give no precipitate after incubation, were subsequently treated with optimal amounts of S-antibody and incubated in the usual manner The precipitin titers in these instances were approximately the same as those obtained in control titrations in which no L-antibody was employed Apparently, the presence of L-antibody, which was presumably combined with the degraded

TABLE III

Inhibition of L- and S-Antibody by Degraded Forms of LS-Antigen

Inhibiting solution	Ratio of inhibiting solution to antiserum	Antiserum	Dilution of dermal filtrate					Saline + test mixture
			1 8	1 16	1 32	1 64	1 128	
None		L	++++	++++	++++	+++	++	
		S	++++	++++	++++	+++	++	
LS heated	1 1	L	+	–	–	–	–	–
	2 1	L	–	–	–	–	–	–
LS heated with alkali	1 1	L	++	++++	+++	++	++	–
	2 1	L	±	+++	+++	++	++	–
	1 1	S	–	–	–	–	–	–
	1/2 1	S	–	–	–	–	–	
	1/4 1	S	±	+++	+	–	–	
	1/8 1	S	++	++++	++++	+++	–	

L-antiserum 1601 and S-antiserum 493 were used in the experiments summarized in this table.

See legend of Table II and text for details of experiment.

L-portion of the antigen, did not interfere with the union of the S-portion with its antibody and subsequent flocculation of the entire aggregate

Experiment 8—A portion of the solution of heated LS antigen used in Experiment 7 was freed of buffer by dialysis and treated with a sufficient amount of sodium hydroxide to bring the final concentration to 0 1 M The material was heated in a water bath at 50°C in a closed vessel for 90 minutes with frequent gentle agitation After neutralization to pH 7 the solution was titered with S-antibody The treatment failed to effect a complete loss of specific precipitability of the solution, for the S-titer, although much diminished, was still 1 320 Accordingly, the procedure was repeated except that 0 05 M concentration of alkali was used Although the first treatment did not result in any change in the appearance of the solution, this second treatment was accompanied by the production of a small amount of insoluble material which, incidentally, was arranged in small, elongated ribbon-like fibers which were white in

reflected light and colorless in transmitted light The insoluble material was removed and the solution tested in dilutions of 1 5 to 1 640 at pH 7 with optimal amounts of S-antibody No precipitation occurred.

An inhibition experiment of the type described in the previous section was now performed L- and S-antisera were treated with varying amounts of alkali heat degraded antigen and subsequently tested for precipitable antibody The results of the experiment are summarized in Table III

It is apparent from the data presented in Table III that as little as 0 1 cc. of the solution of LS-antigen which had been degraded by heat and alkali (approximately 0 15 mg of dried material) was sufficient to inhibit completely the S-antibody in the mixture Furthermore, even ¼ of this amount of antigen was sufficient to inhibit the antiserum slightly On the other hand, 1 0 cc. (1.5 mg) was incapable of inhibiting the amount of L-antibody employed. Thus, degradation of LS-antigen by means of heat and alkali completely destroys the serological activity of the L-portion of the molecule and changes the S-portion of the molecule to a form which no longer precipitates with S-antibody but is capable of inhibiting it. It will be noted that the inhibitory action of the degraded S-portion of the molecule on S-antibody is of a greater order of magnitude than that of the degraded L-portion for its corresponding antibody

Electrophoretic studies on the solution of LS-antigen in Experiment 8 which had been degraded by heat and alkali showed that it contained one electrically homogeneous component with a mobility of $6\ 4 \times 10^{-5}$ cm /sec per volt/cm at pH 7 9 and ionic strength 0 05 The mobility of this substance is close to that of component I in dermal filtrate The two are not identical, however, for the latter failed to inhibit S-antibody Furthermore, the two substances have different solubilities the S-inhibitor is precipitated at pH 4.5 and fails to dissolve appreciably at pH 6.3, but it can be brought into solution between pH 8 and 9, and will then remain in solution when the pH is lowered to nearly 7 0

DISCUSSION

The experiments presented in this paper indicate that all of the serological activity associated with the heat-stable and heat labile soluble antigens of vaccinia are present in a single protein molecule. This differs somewhat from the concept of Craigie and Wishart (5), who considered the two antigens to occur, ordinarily, in the form of a complex which could be dissociated into two separate antigens under certain circumstances

It is possible to state with assurance that various levels of degradation of LS-antigen can be accomplished, leaving either the L- or S part of the molecule in a stage where it can still combine with the corresponding antibody without precipitation The observations on inhibition of L- and S-antibody reported in the previous paper were not sufficiently conclusive to enable us to decide as to the nature and origin of the inhibiting substances On the basis of the

present studies it is clear that they arise directly from the LS-antigen through partial degradation of the molecule

Here it has been shown that the L-portion of the LS-molecule can be degraded by heat without serological alteration of the S-portion In a subsequent paper in this series, it will be demonstrated that by means of enzymatic digestion the S-portion of the molecule can be degraded without altering the serological activity of the L-portion On the basis of this latter observation it is possible to explain directly (2) the results occasionally obtained by other workers (4, 5), which had been interpreted (5) to indicate a dissociation of their LS-complex antigen into two separate components

SUMMARY

Virus-free filtrate, obtained from suspensions of vaccine virus-infected dermal pulp of rabbits and rich in the soluble substances of vaccinia, was shown to contain four distinct components in electrophoresis experiments Electrophoretic and serological observations served as a guide in developing a method for separating these components from one another This method depended upon changes in the solubilities of the components with alterations of pH

Three of the four components appeared to be serologically inert when tested with anti-vaccinia sera All of the L- and S-activity was found to be associated with a single component which was electrically homogeneous at several values of pH and which was homogeneous in the ultracentrifuge

This single substance, designated as LS-antigen, precipitates in equal titers with optimal amounts of L- and of S-antibody and is completely removed from solution by absorption with either antibody

The LS-antigen of vaccinia appears to be a protein molecule with two antigenically distinct parts, L and S Heating modifies the L-portion in such a manner that the substance no longer precipitates with L-antibody, this degraded antigen still combines with L-antibody, as is shown by inhibition tests, and still precipitates with S-antibody Similarly, treatment with heat and dilute alkali modifies the S-portion of LS-antigen so that it combines but does not precipitate with S-antibody, and at the same time all recognizable immunological properties of the L-portion are destroyed

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THE EFFECT OF VARIOUS DIETS ON THE LIVER DAMAGE CAUSED BY EXCESS CYSTINE

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Previous reports (1–4) have described the rapid production of portal necrosis and hemorrhage in the livers of rats fed large amounts of *l*-cystine In addition, more prolonged feeding of *l*-cystine causes the development of cirrhosis of the liver (4) The present paper is a study of the effects of variation in diet on the development of these liver lesions

Methods

Six different diets containing 10 gm of *l*-cystine per 100 gm of food (subsequently referred to as "10 per cent cystine") and 3 different diets containing 5 per cent cystine" were fed to male albino rats, 6 weeks of age The protein of the 'synthetic' diets was supplied by casein and yeast, the fat by lard and cod liver oil or by butter, and the carbohydrate by sucrose. The relative amounts of these ingredients were varied in the different diets Table I shows the composition of the diets the average daily food consumption per rat and the number of rats fed each diet. An additional series of rats was fed 5 and 10 per cent cystine in the McCollum stock diet.

These diets permitted a comparison of the effects of different protein, fat, carbohydrate, and yeast concentrations on the hepatic lesions produced by 10 per cent cystine. It was also possible to compare the effects of different kinds of fat namely butter *versus* lard and cod liver oil as well as the effects of synthetic diets *versus* the natural food McCollum stock diet. In the 5 per cent cystine group the McCollum stock diet was compared with a low protein, high lard, and cod liver oil diet The effect of the addition of 1 per cent choline to this low protein, high fat diet was also examined

The technique employed for classifying the liver lesions was similar to that described in a previous paper (4) Hemorrhage and necrosis were graded 0–4, each unit indicating involvement of ¼ of the area of the section Portal fibrosis and bile duct proliferation were also graded 0–4 each unit indicating involvement of ¼ of the portal areas. In cases where there was any question as to the presence or absence of portal fibrosis, the Mallory connective tissue stain was employed. Hypertrophy and vacuolization of the liver cells were graded on the same basis as hemorrhage and necrosis Estimation of the number of mitotic figures was made by counting the

number seen in 3 to 10 low power fields (magnification × 100) and obtaining the average per low power field.

The fat content of the livers was determined by the method previously described (4).

TABLE I

Per Cent Composition of Diets

	10 per cent cystine						5 per cent cystine		
Diet	10HL	10HB	10LF	10HP	10HY	10 S	5HL	5HLC	5 S
Type	Low protein High lard	Low protein High butter	Low fat Low protein	Low fat High protein	Low fat High yeast	McCollum stock	Low protein High lard	Low protein High lard + Choline	McCollum stock
l-Cystine	10	10	10	10	10	10	5	5	5
Salt mixture	4	4	4	4	4	*	4	4	*
Brewer's yeast	5	5	5	5	20		5	5	
Casein	5	5	5	40	5		5	5	
Lard	20	—	3	3	3		20	20	
Cod liver oil	5	—	2	2	2		5	5	
Butter	—	25	—	—	—		—	—	
Sugar	51	51	71	36	56		56	55	
Choline HCl	—	—	—	—	—		—	1	
No. of rats	29	14	10	10	10	12	15	15	15
Intake, gm./rat	3.6‡	3.8	3.0	3.0	3.3	3.8	6.1§	6.8	6.1

The number of rats fed each diet and the average daily intake of food are indicated below the dietary system. The *l*-cystine and the choline were obtained from the Eastman Kodak Co., Rochester, New York.

* The McCollum stock diet (5) has the following composition in parts per cent: bran 67.5, whole milk powder 15, casein 10, butter 5, NaCl 1.5, and Na_2CO_3 1.0.

‡ Average of 10 rats only.

§ Average of 7 rats only.

RESULTS

In Table II are summarized the per cent mortality, the incidence per cent of the liver lesions, and the average fat content of the livers of rats fed the several diets described. These features have been divided into 2 periods, namely, those occurring before and those after 2 weeks of the dietary regimen. The grouping of the data followed the general scheme outlined in Table I except that the groups fed 10 per cent cystine in the 3 low fat diets (low protein, high protein, and high yeast) were combined. These 3 groups showed no significant differences among themselves in their response to the 10 per cent cystine, with one exception which will be mentioned later. The mortality of the 10 per cent cystine groups during the first 2 weeks was calculated on the basis of the number of animals that died and that survived throughout the

period The animals sacrificed during these 2 weeks were not considered in the calculation

TABLE II

Effect of Various Diets Containing 10 Per Cent or 5 Per Cent Cystine on Per Cent Mortality Per Cent Incidence of Liver Lesions and Liver Fat Content

	No. of rats		Per cent mortality		Per cent incidence liver lesions						Per cent fat content			
					Hemorrhage		Necrosis		Cirrhosis					
Days on diet.	2-14	15+	2-14	15+	2-14	15+	2 14	15+	2-14	15+	2 14		15+	
											Average	Range	Average	Range
10 per cent cystine in McCollum stock	10	2	78	—	20	—	50	—	41	12	2 2 (4)*	1 6- 3 1	2 2	2 1- 2 2
10 per cent cystine in low fat	29	1	97	—	21	—	86	—	52	11	3 4 (20)	1 7-13 2	4 9	
10 per cent cystine in low protein high lard	22	7	71	—	73	29	77	71	14	71	4 0 (11)	2 5- 7 9	15 3 (3)	13 1-19 1
10 per cent cystine in low protein high butter	14	0	100	—	71	—	93	—	29	—	7 1 (12)	2 5-15 2		
5 per cent cystine in McCollum stock	5	10	0	0	0	0	60	20	80	50	1 4	1 1- 2 0	2 6	1 4- 5 0
5 per cent cystine in low protein, high lard	5	10	0	0	0	20	0	70	0	40	13 6	4 8-17 4	23 5	7 6-42 3
5 per cent cystine in low protein high lard + choline	5	10	0	0	0	0	20	20	40	30	4 3	1 6-11 4	2 4	0 8- 4 6

* The numbers in parentheses represent the number of livers in the group analyzed for fat In all other instances the entire group was analyzed.

The average amount of a specific lesion in a particular group of rats is equal to its percentage incidence multiplied by its average severity (calculated from data derived as described under Methods) On this basis, the average amount of hemorrhage, necrosis, and cirrhosis was calculated for the groups of rats that died or were sacrificed during the first 2 weeks on the various diets

containing cystine In Fig 1, these values along with the average liver fat content are graphically presented

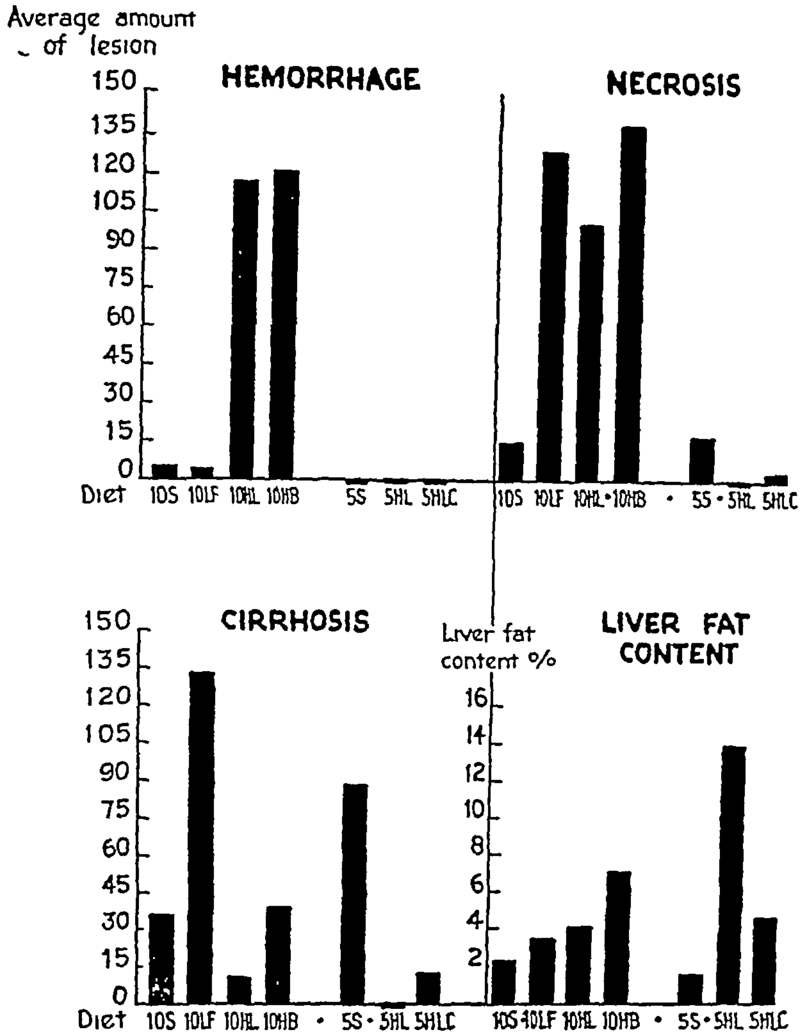

FIG 1 Effect of different diets on liver lesions due to excess dietary cystine First 2 weeks only Ordinates represent average percentage incidence and average severity of liver lesion Abscissa lists different diets, 10 = 10 per cent cystine, 5 = 5 per cent cystine, S = stock, LF = low fat, HL = high lard, HB = high butter, HLC = high lard and 1 per cent choline

Effect of Diets on the Course and Liver Lesions Due to 10 Per Cent Cystine

These data show that the diet modified the mortality rate and the character of the liver lesion resulting from 10 per cent cystine If the McCollum stock

diet, the natural food diet, is considered as the basis for comparison, the following results are apparent

1 A low fat synthetic diet, regardless of the casein or yeast content was deleterious This was indicated by a greater early mortality (only one of 30 rats lived more than 8 days) and by more necrosis and cirrhosis (Fig 1) However, the incidence and severity of hemorrhage were not different.

2 A diet *high* in fat, consisting of lard 20 per cent, and cod liver oil 5 per cent had some protective action This was indicated by a slightly lower mortality and a slower development of cirrhosis (bold faced figures, Table II) On the other hand, the incidence and extent of hemorrhage were far greater than that in the previous groups (McCollum stock and low fat diets) Necrosis was the same as in the low fat diets but greater than was observed in the McCollum diet.

3 The protective action of the high fat diet (lard 20 per cent and cod liver oil 5 per cent) was not due to fat alone, because 25 per cent butter substituted for the lard and cod liver oil, was followed by the most severe and harmful reaction to cystine The mortality was greatest, the animals died in a shorter time (none survived more than 6 days), and hemorrhage and necrosis were most severe In spite of the shorter survival period on this diet, these animals had much more cirrhosis in this 6 day interval than those on the 20 per cent lard and 5 per cent cod liver oil had in a 14 day interval

Effect of Diets on the Liver Lesions Due to 5 Per Cent Cystine

Cystine was fed as 5 per cent of 3 different diets the McCollum stock diet, the low protein, high lard, and cod liver oil diet, and the latter diet plus 1 per cent choline. There were 15 rats in each group As none of these animals died, rats from each group were sacrificed after periods of from 3 to 42 days of cystine feeding Five from each group were sacrificed during the first 2 weeks and 10 subsequently

In general, the incidence and severity of the liver lesions were less than among rats fed 10 per cent cystine (Table II, Fig 1) although the character of the lesion was the same Portal hemorrhage occurred in only 2 of 45 rats fed 5 per cent cystine Necrosis in the portal areas, when present, involved only a few cells.

Portal cirrhosis was present in each group and the incidence was modified by the diet (Table II and Fig 2) Only 5 animals in each group were sacrificed during the first 2 weeks. There were insufficient data, therefore, to ascertain that the rate of development of cirrhosis was influenced by the diet. However, the available data indicate that the rate of development and the incidence of cirrhosis were related, that is, a rapid appearance of cirrhosis was associated with a high incidence of the lesion Comparison of the 15 animals in each of the groups with those fed the 5 per cent cystine McCollum stock diet revealed the following with respect to the incidence of cirrhosis

1 On the low protein, high lard, and cod liver oil diet the incidence was 4 of 15 as compared with 9 of 15 on the McCollum stock diet, that is, about one-half During the first 2 weeks the incidence was 0 of 5 as compared with 4 of 5 In other words, the low protein, high lard diet had a protective action against the development of cirrhosis produced by either 5 per cent or 10 per cent cystine

2 Addition of 1 per cent choline to the low protein, high lard, and cod liver oil diet did not significantly alter the protective effect of this diet, that is, 5

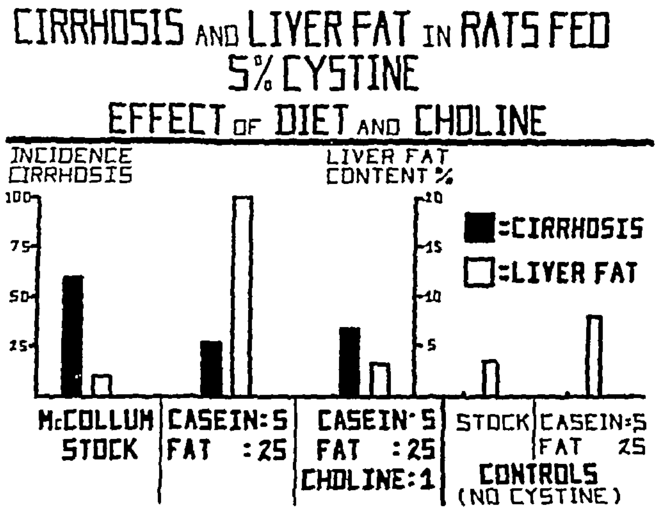

FIG 2 There is no correlation between severity of fatty infiltration and cirrhosis in rats fed 5 per cent cystine Choline, which prevented the fatty infiltration due to the diet, did not inhibit the development of cirrhosis The incidence of cirrhosis was greatest on the McCollum diet, while the fatty infiltration was least

of 15 fed choline had cirrhosis as compared with 4 of 15 on the diet without choline

Effect of Diet on the Fat Content of the Livers of Rats Fed Excess Cystine

The average fat content of livers of 12 rats on the McCollum diet without cystine was 3 5 per cent (range 3 0–4 5) None of these control livers showed evidence of necrosis or cirrhosis The average liver fat content of rats fed 5 per cent or 10 per cent cystine in the McCollum stock diet was 2 2 per cent Although the livers of a few rats fed 10 per cent cystine in the low fat diets had slight increases in fat content, the average value was within the normal limits described above (Table II)

In contrast to the above, the average liver fat content of 8 rats on a low protein, high lard, and cod liver oil diet without cystine for 3 to 40 days was 8 1 per

cent (range 4.2–13.3) No liver lesions other than fatty infiltration occurred in this group Addition of 10 per cent cystine to this low protein, high fat diet resulted in a further increase in the liver fat content in rats that survived more than 2 weeks The 5 per cent cystine diet, however, resulted in an earlier and greater fatty infiltration of the liver than 10 per cent cystine (Table II) It is likely that this is related to the total food intake rather than to cystine alone, for, as Table I indicates, the animals that received 5 per cent cystine ate twice as much food as those receiving 10 per cent cystine Therefore, both groups ingested the same amount of cystine, but the 5 per cent group ate twice as much fat.

The fatty infiltration observed with 5 per cent cystine in the high fat diet was inhibited by the addition of 1 per cent choline

The Relationship of Fatty Infiltration and Cirrhosis of the Liver Due to Excess Dietary Cystine

The results described above offer an opportunity to determine any correlation between liver fat content and the development of cirrhosis in rats fed excess cystine. In these experiments, the diet modified both of these characteristics and if there were any causal relationship between them, the fatty infiltration and the cirrhosis should have shown some correlation

Fig 2 shows the incidence of portal cirrhosis of the liver and the average liver fat content of groups of 15 rats fed 5 per cent cystine in the McCollum stock diet, a low protein-high fat diet and a low protein high fat diet plus 1 per cent choline. Cirrhosis was found in 60 per cent of the McCollum stock diet group and the average liver fat content was only 2.2 per cent Although 1 per cent choline added to the low protein, high lard, and cod liver oil diets greatly lowered the liver fat content, *i.e* from 20 7 per cent without choline to 3 0 per cent, it did not influence the incidence of cirrhosis which was approximately 30 per cent, with or without choline

Fig 3 is a scatter chart of the fat content and the severity of cirrhosis in the livers of rats fed excess cystine in the various diets

No correlation between the liver fat content and the incidence or severity of cystine cirrhosis was evident from the above data

Other Findings

Hypertrophy and vacuolization of the liver cells were found in each group In general the incidence and severity of these features paralleled the degree of fatty infiltration However, in a number of instances, the liver cells had hydropic vacuoles and the liver fat content was normal or low In these cases, the vacuoles did not take up Sudan III.

All livers were studied for the presence of mitotic nuclei In contrast to the other rats, many mitotic nuclei were found only among the rats fed cystine

as 10 per cent of the low fat, high *protein* diet Among the rats fed 5 per cent cystine, no mitoses were seen in the livers of the stock diet group, but were observed in a few of those receiving low protein and high fat, with or without 1 per cent choline

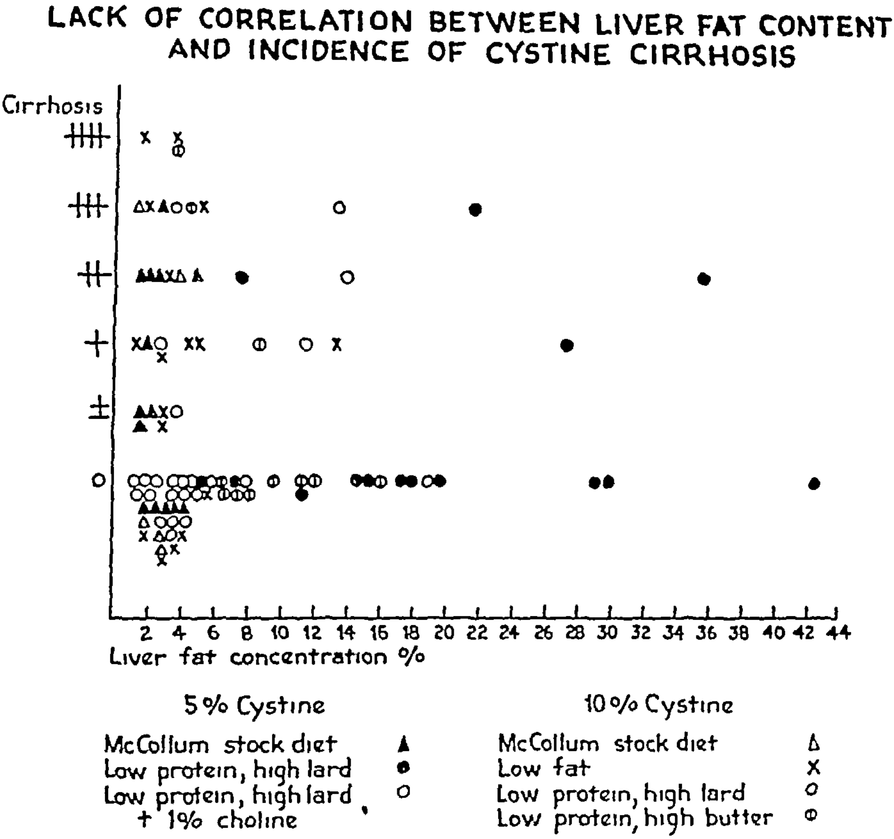

FIG 3 Scatter chart showing degree of cirrhosis and fat content of the livers of rats fed excess cystine in various diets For cirrhosis ± = fibrosis of less than ¼ of the portal areas, + = fibrosis in ¼ of the portal areas, ++ = fibrosis in ½ of the portal areas, and so on

In a few instances, where severe liver damage had resulted from excess dietary cystine, enlargement of the spleen with congestion and fibrosis was noted

Of 130 rats fed cystine as 5 or 10 per cent of various diets, hemorrhagic kidneys were found but once

Summary of Results

Hemorrhage in the liver was most severe with a high fat intake, whether the fat was lard and cod liver oil or butter *Necrosis* was more severe with synthetic diets, regardless of the proportions of fat, protein, carbohydrate, or yeast, than with the natural food diet. *Cirrhosis* was less severe during the first 2 weeks on the high lard and cod liver oil diet than on the high butter, or low fat, or the McCollum diets *Fatty infiltration* in the livers of rats fed excess cystine was also modified by diet. There were slight increases in the liver fat content of only a few rats on the McCollum stock and low fat diets Marked increases were noted in the low protein, high fat groups. This increase in liver fat was prevented by 1 per cent choline The number of *mitotic nuclei* in the liver cells was greatest among the rats fed the high protein, low fat diet. In other words, not only is the degree of cystine damage to the liver affected by diet, but the pathogenesis of the lesion is also dependent on the diet.

DISCUSSION

The differences in liver lesions produced by cystine fed in various diets were related to differences in the diets and not to differences in consumption of cystine The average daily food consumption of the rats fed 10 per cent cystine was essentially the same with all diets (Table I) The food consumption of rats fed 5 per cent cystine was approximately twice that of rats fed 10 per cent cystine, so that all groups consumed the same amount of cystine

It is possible, though not probable, that there were differences in the amounts of cystine absorbed from the gastrointestinal tract on the various diets In this connection it should be noted that Wilson (6) found that rats could absorb cystine from the gastrointestinal tract at the rate of 30 mg per hour, or 720 mg in 24 hours In the present experiments, the greatest amount of cystine consumed by an individual rat in 24 hours was 630 mg , while the average intake for the groups of rats on the different diets varied from 300 to 380 mg in 24 hours.

The liver lesions produced by cystine are related to the proportion of cystine in the diet but not to the amount of cystine ingested This is illustrated by the fact that there were differences in the liver lesions between rats fed 5 per cent and 10 per cent cystine (Table III), although both groups ingested the same amount of cystine, the food consumption of rats fed 5 per cent cystine being twice that of rats fed 10 per cent cystine (Table I)

It has been reported that high dietary fat enhances the deleterious effect of various hepatotoxins (7–12) or may produce cirrhosis (19, 20) The present studies indicate that certain fats such as lard and cod liver oil, when fed as 25 per cent of the diet, have some protective effect against liver damage caused

by excess cystine, while butter does not In liver damage due to selenium, it has been found that a diet containing 39 per cent beef fat and 2 per cent cod liver oil protected against the development of cirrhosis of the liver (13)

Most of the reported studies state that protein (8–12) and yeast (14) are protective against certain liver poisons In the present experiments, liver damage resulting from 10 per cent cystine in low fat diets was not influenced by 5 or 40 per cent casein or by 5 or 20 per cent brewer's yeast The studies reported in this paper agree with the findings that yeast *per se* has no protective action on carbon tetrachloride liver damage when the food intake is controlled (15)

György and Goldblatt (16) have found necrosis and cirrhosis of the liver in rats fed, for 100 or more days, a diet devoid of vitamin B, but supplemented with thiamine, riboflavin, pyridoxine, and pantothenic acid The diet was also low in protein (casein 10 per cent) and high in fat (lard 20 per cent, cod liver oil 2 per cent) The addition of small amounts of cystine increased the severity of liver damage while choline prevented it Similar findings were reported by Blumberg and Grady (17) More recently, Webster (18) has reported necrosis and cirrhosis in rats fed low protein, high fat diets which also contained adequate amounts of brewer's yeast These lesions were made more severe by the addition of small amounts of cystine and were prevented by choline The present experiments show that the liver damage caused by 5 per cent cystine fed in a 5 per cent casein, 20 per cent lard, and 5 per cent cod liver oil diet was not prevented by 1 per cent choline, although the fatty infiltration of the liver was markedly inhibited (Fig 2)

This difference in the response to choline is a distinction between the pathogenesis of the liver lesion produced by cystine and that caused by low protein high fat diets (16–20) Other differences are found in the liver lesions as well as in the rate of their development On the cystine diet, the lesions originated in the portal areas while those described by György and Goldblatt (16) showed diffuse degeneration The cirrhosis caused by cystine may appear within a week, while under the conditions summarized above (16–18) about 4 months on the diet were required for the appearance of cirrhosis The cirrhosis caused by cystine was associated with considerable bile duct proliferation (4) and not necessarily with fatty infiltration That found under conditions mentioned by others (16–20) had considerable fatty infiltration and little bile duct reaction, even when small quantities of cystine were added to the diet (16)

SUMMARY AND CONCLUSIONS

1 The effect of 9 different diets on the liver lesions resulting from excess dietary cystine has been studied in 130 rats

2 The incidence and severity of each of the following liver lesions were

varied by changes in the composition of diets containing 5 or 10 per cent cystine

(*a*) *Hemorrhage* was least severe with low fat diets

(*b*) *Necrosis* was most severe with synthetic diets

(*c*) *Cirrhosis* was delayed by a diet high in lard, 20 per cent, and cod liver oil, 5 per cent, but not by a diet high in butter, 25 per cent.

(*d*) *Fatty infiltration* was found consistently only with low protein, high fat diets

In other words, the pathogenesis of the liver lesion due to excess dietary cystine can be modified by diet.

3 In the presence of cystine as 5 per cent of a low protein, high fat diet, 1 per cent choline inhibited fatty infiltration but did not protect the liver against damage by cystine

4 In these experiments there was no apparent correlation between fatty infiltration of the liver and the incidence or degree of cirrhosis

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LIVER DAMAGE AND URINARY EXCRETION OF SULFATE IN RATS FED *l* CYSTINE, *dl* METHIONINE, AND CYSTEIC ACID

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It has been reported that rats fed *l*-cystine as 5 or 10 per cent of the diet develop portal necrosis and hemorrhage of the liver (1–4) In addition, more prolonged feeding of *l*-cystine results in portal cirrhosis of the liver (4) It has been shown that the metabolism of *l*-cystine (5) and *dl*-methionine (6) results in the urinary excretion of the greater part of the sulfur contained in these amino acids as sulfate Schmidt and Clark (7), however, have demonstrated that the sulfur of cysteic acid is not oxidized by the animal organism but is excreted in the neutral sulfur fraction of the urine

If the formation and excretion of large amounts of sulfate resulting from the metabolism of *l*-cystine were the cause of the liver lesions previously described, then *dl*-methionine fed in excess amounts should also result in liver damage Cysteic acid on the contrary, should not have this effect The present paper is a report of experiments in which this hypothesis was put to the test

Methods

The sulfur-containing amino acids, *l*-cystine and *dl* methionine were obtained from the Eastman Kodak Co The cysteic acid was prepared from *l*-cystine by the bromine oxidation method and was neutralized by NaOH before administration

The sulfur-containing compounds were thoroughly mixed into either the McCollum stock diet or a low protein, high fat diet of the following composition in grams per 100 gm. of food casein 5 butter 25 brewers yeast 5 salt mix 4 and sugar *ad* 100 These diets were then fed to albino rats and the daily intake noted. At intervals 24 hour urine collections were made for urinary sulfur partitions (8) as well as ammonia determinations. In addition blood for CO_2-combining power values was obtained at the time of sacrifice. Finally, microscopic sections of the liver of each rat were studied for the presence or absence of necrosis and cirrhosis. These features, when present, were graded on the basis of + to ++++ (4)

The protocols of the 15 rats studied are presented in Table I. The urine collections periods for rats 9, 14, and 15 were divided between the first and last days of

the experiment In all other instances, the collection periods were made consecutively during the last days on the experimental data

RESULTS

The intake of sulfur and the total sulfur, sulfate sulfur, and ammonia nitrogen urinary excretion figures are given in Table II These figures represent the averages of the values obtained for each rat in the number of collection periods indicated in Table I, and are recorded as milligrams per 100 gm of rat per 24

TABLE I

Weight, Diet, Days on Diet, Number of Balance Periods, and Outcome of Rats Fed Sulfur Containing Compounds

Rat No	Weight at start	Basal diet	Sulfur compound fed: Compound	Sulfur compound fed: Per cent of diet	Days on diet	No of collection periods	Outcome
	gm						
1	184	Low protein, high fat	*l* Cystine	10	3	3	Died
2	72	" " " "	"	10	4	4	Sacrificed
3	96	" " " "	"	10	3	3	"
4	190	" " " "	"	10	4	4	"
5	79	" " " "	"	5	34	6	"
6	90	" " " "	"	5	34	6	"
7	116	" " " "	"	5	38	4	"
8	92	McCollum stock	"	5	38	4	"
9	90	" "	"	5	38	6	"
10	71	Low protein, high fat	*dl* Methionine	12 8	6	5	"
11	52	" " " "	"	6 4	6	5	Died
12	224	McCollum stock	"	10	12	2	Sacrificed
13	278	" "	"	10	12	2	"
14	112	McCollum stock	Cysteic acid	15	10	2	"
15	71	" "	" "	15	15	3	"

hours The per cent of sulfate neutralized by ammonia and the blood CO_2-combining power values are also given in Table II

The urinary excretion of sulfur was studied in 6 of the rats on the basal low protein, high fat diet before the addition of the sulfur compounds The average excretion in 12 control periods was 6 7 mg of total sulfur and 3.3 mg of sulfate sulfur per 100 gm rat per 24 hours The corresponding figures for 5 control periods in 3 rats on the basal stock diet were 11 2 mg and 7 3 mg The excretion figures for sulfur given in Table II were not corrected for these control values as the food intake and body weight declined during the periods of *l*-cystine and *dl*-methionine feeding From the available data it was im-

possible to calculate how much of the sulfur excreted during the experimental periods was derived from exogenous and how much from endogenous stores. In any case, the control values were relatively small when compared to the experimental figures and the results do not appear to be invalidated by excluding the control values from the calculations

From Table II it may be seen that the rats fed excess *l*-cystine or *dl*-methionine excreted relatively huge amounts of sulfate sulfur while the rats fed

TABLE II

Summary of Chemical Data of Rats Fed Sulfur-Containing Compounds

Rat No	Sulfur compound fed			Urine				Blood CO_2-combining power
	Compound	Per cent of diet	Intake sulfur*	Total sulfur*	Sulfate sulfur*	Ammonia nitrogen	SO_4 neutralized by NH_4	
							per cent	*vol per cent*
1	*l* Cystine	10	94	32	16	—	—	—
2		10	126	79	40	—	—	55
3		10	160	86	43	31	82	36
4		10	57	37	18	15	92	40
5	"	5	76	68	42	25	71	41
6		5	49	53	29	17	65	43
7		5	90	102	62	49	80	36
8		5	91	90	67	48	81	37
9		5	83	86	54	47	92	—
10	*dl* Methionine	12 8	85	66	52	32	70	—
11	"	6 4	42	42	38	31	90	—
12		10	40	36	27	14	61	42
13		10	31	22	17	7	44	37
14	Cysteic acid	15	150	88	15	21	164	—
15		15	211	151	18	31	138	—

* Figures in these columns represent mg per 100 gm. of rat per 24 hours.

cysteic acid formed much smaller amounts of sulfate These results are in accord with many other published studies of a similar nature (5–7) Enough sulfate was excreted by the rats fed *l*-cystine and *dl*-methionine to require large amounts of base for neutralization It might be expected that this, continued over a considerable length of time, would be a serious drain on the rats' reserves of base The blood CO_2-combining power determinations, however, showed no or moderate decreases from normal values This is probably explained by the capacity of these animals to form large amounts of ammonia As shown in Table II, sufficient ammonia was present in the urine of rats fed

l-cystine or *dl*-methionine to neutralize as much as 92 per cent of the sulfate excreted

The degree of necrosis and cirrhosis present in the livers of the rats is recorded in Table III Hepatic necrosis and/or cirrhosis was noted in each of the rats fed *l*-cystine and cysteic acid Although the livers of the rats fed *dl*-methionine were very small and microscopic examination revealed atrophy of the liver cells, necrosis and cirrhosis were not found Twenty additional rats were fed *dl*-methionine at excessive levels in various diets No cirrhosis was found in any of these, while a few isolated necrotic cells were seen in the liver of one rat only A more detailed description of the effects of feeding *dl*-methionine and cysteic acid to rats will be given in another communication

TABLE III

Liver Damage in Rats Fed Sulfur-Containing Compounds

Rat No	Compound fed	Per cent of diet	Liver lesions	
			Necrosis	Cirrhosis
1	*l* Cystine	10	+++	0
2	"	10	+	+
3	"	10	++	+
4	"	10	0	+
5	"	5	+	+++
6	"	5	0	++
7	"	5	0	+++
8	"	5	+	++
9	"	5	0	+
10	*dl*-Methionine	12 8	0	0
11	"	6 4	0	0
12	"	10	0	0
13	"	10	0	0
14	Cysteic acid	15	+	++
15	" "	15	+	++

SUMMARY

1 Rats fed *l*-cystine at excessive levels excrete large amounts of sulfate sulfur in the urine and develop liver necrosis and cirrhosis

2 Rats fed *dl*-methionine at excessive levels excrete large amounts of sulfate sulfur, but do not develop liver necrosis and cirrhosis

3 Rats fed cysteic acid at high levels excrete relatively small amounts of sulfate, but do develop liver necrosis and cirrhosis

4 In conclusion, there is no apparent correlation between the amount of

sulfate formed and the development of liver damage in rats fed *l*-cystine, *dl*-methionine, and cysteic acids

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CELL STATE AS AFFECTING SUSCEPTIBILITY TO A VIRUS

Enhanced Effectiveness of the Rabbit Papilloma Virus on Hyperplastic Epidermis

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Plates 2 and 3

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The effects of some viruses are known to be altered when they act upon cells which have been previously rendered abnormal The virus of herpes simplex induces lesions resembling those of herpes zoster in rabbit skin altered by tar (1), and ultraviolet irradiation of rabbit skin modifies the local susceptibility to inoculation with vaccine virus (2) The Shope papilloma virus (3) elicits carcinomas forthwith, as well as various papillomas of unusual sort, when it is brought into association with the epidermis of domestic rabbits, which has been tarred for some weeks (4) The experiments reported in the present paper were undertaken to learn whether preliminary alterations of rabbit epidermis would provide a more favorable soil for the demonstration of the papilloma virus Often no virus can be got from the papillomas of domestic rabbits directly produced therewith though its presence in the growths is readily demonstrable serologically (5, 6) as also in the cancers deriving from them (7)

Material and Methods

Papilloma virus was obtained from the naturally occurring growths of cottontail rabbits which had been preserved in 50 per cent glycerin Locke's solution at about 4 C Weighed portions of the tissue were passed through several changes of saline ground with sand, and suspended in 10 to 20 volumes of 0 9 per cent saline. The crude extracts thus obtained were spun in an angle-head centrifuge at about 3500 R.P.M. for 5 minutes and the supernatant fluids were again spun at about 4500 R.P.M for 20 minutes They were now clear amber and were usually highly infectious as such and after filtration through Berkefeld V candles For test they were inoculated intradermallv or rubbed into the skins of adult domestic rabbits of the gray brown (agouti) breed or cottontail rabbits (genus *Sylvilagus*) after scarification with sand paper according to a method already described (5, 6) The character of the papillomas arising was recorded at frequent intervals from the 8th day to about the 42nd day after inoculation according to a standard scale ++++ = confluent papillomatosis, +++ = semiconfluent papillomatosis, ++ = many discrete papillomas, + = 5 to 15 papillomas ± = 2 3 or 4 papillomas, ± = 1 papilloma, 0 = negative

To conserve space, the readings of only a few days are given in the tables They are representative of the findings as a whole

Influence of Carcinogenic Agents to Alter Skin Susceptibility

In a first experiment the results of rubbing a suspension of papilloma virus into scarified skin were compared with those consequent on infiltrating tarred rabbit ears with the suspension by way of a marginal vein

Experiment 1 —The ears of fourteen normal cottontail rabbits were tarred over the inner and outer surfaces on three occasions at intervals of 3 or 4 days This amount of tarring caused the ears to become acutely inflamed, thickened, and moist, but elicited no tumors A strong rubber band was placed about the base of the ear, to stop the circulation, and 5 cc. of a virus filtrate (W R 1-28) diluted from 1 500 to 1 480,000,—in terms of the papilloma material originally extracted,—was then slowly injected into a marginal vein of the ear The rubber bands were removed 5 minutes after injection The ears of each rabbit received different dilutions of the virus 0 2 cc. of the same dilutions was then rubbed into scarified areas of normal skin on the abdomens of the same rabbits

The results can be briefly summarized Six of the rabbits proved resistant to infection, only a few growths arising from either the tarred or the normal epidermis Eight rabbits were susceptible and in every one many more papillomas developed from the tarred epidermis than from the normal skin inoculated with the same dilution of virus The ratio of the number of papillomas on the tarred skin to that on the normal skin was as high as 135 or more to 1 Furthermore papillomas were elicited in the tarred ears by a considerably higher dilution of virus In one instance a dilution of 1 80,000 elicited papillomas on the tarred epidermis, yet 1 500 failed to do so on the untreated skin In five animals papillomas appeared on the tarred ears and none on the scarified skin in response to the same dilution of virus All of the growths were benign papillomas

The findings indicate that rabbit skin to which tar has been applied a few times is more susceptible to infection with the papilloma virus than normal scarified skin The objections can be raised, however, that the total amount of virus introduced into the tarred ears was greater than that rubbed into the skin of the abdomen and that neither the skin situations nor the methods of inoculation were comparable To control these variables an experiment was done next in which virus was rubbed into areas of skin, some of which had been tar-treated while others had been left untreated prior to scarification As enlarging the test, other areas which had been treated previously with methylcholanthrene or benzpyrene were inoculated with virus

Experiment 2 —The hair was clipped from twelve rectangular areas of skin measuring about 3 × 5 cm on the abdomens of four domestic rabbits Small hairy strips were left between the areas which were in three anteroposterior rows of four areas each One area of each row was painted with a carcinogenic tar, another with 0 3

per cent methylcholanthrene in benzene, and a third patch with 0 3 per cent benzpyrene in benzene.[1] Small camel's hair brushes were used to apply the agents. The remaining area of each row served as control The situations of the control and treated areas were varied from animal to animal. After each treatment they were covered individually with sterile gauze pads, moored with adhesive to prevent spread of the reagents, after which a gauze pad and many tailed binder were put over all. The skin treatments were repeated twice per week for 2 weeks. They elicited no tumors

Two days after the last treatments the patches were greatly changed. Those that had been tarred showed on stripping an acutely inflamed and thickened epidermis covered with a moist yellowish brown scurf The methylcholanthrene-treated skin was also inflamed and greatly thickened, but less so, and was covered with a dry brown scurf The benzene treated skin had a similar appearance To learn the nature of the changes a narrow slice of a normal area and of each of the treated patches was taken under ether anesthesia from each rabbit, fixed in acid Zenker solution and stained with eosin and methylene blue.

Immediately after the biopsies the areas were scarified with sandpaper and a 0 5 per cent virus extract (W R. 1 28) was rubbed into one row of the normal and treated skin areas, and another similarly prepared extract (W.R. 11-69) into another row The treated areas proved more difficult to scarify than the normal skin, the tar treated areas especially, because of the moist adherent scurf Immediately after inoculation the patches were dried in a blast of warm air and bandaged as before.

The results of the experiment are shown in Table I The findings with the two virus extracts can be considered together On the 10th day after virus inoculation, discrete papillomas had appeared on most of the methylcholanthrene treated skin areas and on a few of the benzpyrene- and tar treated areas No growths were visible on the normal skin at this time By the 15th day the papillomas on the treated skin areas had greatly increased in number and had enlarged rapidly, many of them now being semiconfluent papillomatous masses The normal skin areas, on the other hand showed relatively few papillomas and they were small and discrete. On the 20th day most of the treated skin areas showed large, vigorous confluent or semiconfluent papillomatous masses up to 1 0 cm high while the growths on the normal skin were still discrete, in some instances just appearing and only occasionally were as much as 0.3 cm. high The papillomas on the treated patches were in many instances dark gray while those on the healed normal skin of the same animal were pink or light gray All were ordinary virus-induced papillomas

The experiment shows (Table I) that a few preliminary applications to rabbit skin of tar, benzpyrene, or methylcholanthrene greatly enhance the susceptibility of the skin to papilloma virus infection Growths appeared earlier in the treated skin, were more numerous, tended to be more pigmented

[1] The tar came from the Ostergasfabrik of Amsterdam, and was the generous gift of Dr Karl Landsteiner It has been employed in much work in this laboratory The methylcholanthrene was obtained from the Eastman Kodak Company and the benzpyrene from Hoffmann La Roche Inc.

and grew more rapidly, forming large papillomatous masses at a time when the growths in the normal skin were just appearing or were still small and discrete Similar findings were obtained in another experiment when virus was inoculated into normal and methylcholanthrene-treated skin by means of a tattoo machine (Figs 6 and 7)

TABLE I

Susceptibility to the Papilloma Virus of Normal Skin and Skin Treated with Carcinogenic Agents

Papilloma virus extract	Agent applied to skin before virus inoculation*	Pathogenicity tests											
		10th day				15th day				20th day			
Test rabbits		a	b	c	d	a	b	c	d	a	b	c	d
No													
	Normal skin	0	0	0	0	+	±	±	+±	++	++	+±	++±
W.R 1 28	Tar	0	0	0	+	+±	+++	+	+++	+++±	++++	+++	++++
(0.5 per	Benzpyrene	+	0	0	$\underline{\pm}$	++±	+++	++	+++±	+++	+++±	++++	++++
cent)	Methylcholanthrene	+±	±	$\underline{\pm}$	+±	+++	+++	+++	+++±	++++	++++	++++	++++
	Normal skin	0	0	0	0	+	±	±	+±	++	+±	+	++
W R 11-69	Tar	0	0	0	0	±	++±	±	+++	++	++++	++	+++±
(0.5 per	Benzpyrene	0	0	0	$\underline{\pm}$	++	++±	+	++±	+++±	++++	+++	+++±
cent)	Methylcholanthrene	+	±	0	±	+++	+++	++	+++	++++	++++	+++	+++±

* Benzpyrene and methylcholanthrene (0 3 per cent in benzene) and tar applied twice per week for 2 weeks Virus inoculated 2 days after last treatments

++++ = confluent papillomatosis
+++ = semiconfluent papillomatosis
++ = many discrete papillomas
+ = 5 to 15 papillomas
± = 2, 3, or 4 papillomas
$\underline{\pm}$ = one papilloma
0 = negative

Effect of Non-Carcinogenic Agents to Alter Skin Susceptibility

The agents used in the preceding experiment were potent carcinogens, but were employed for so brief a period that they elicited no tumors A test was next done to find whether non-carcinogenic agents, inflammatory for rabbit skin, would have the same effect

Experiment 3—Twelve skin areas on the abdomens of four domestic rabbits were clipped as in Experiment 2 Three on each animal were painted with benzene as such and three others with 0 3 per cent methylcholanthrene in benzene three times per week for 2 weeks Three more areas were exposed to 1500 r of x-ray irradiation 48 hours before virus inoculation The rabbits were protected by flexible lead foil during irradiation, leaving only the areas exposed, and the rays came from a single tube run at 5 milliamperes and at a peak voltage of 135 kilovolts, without filtration

The distance from tube to skin was 50 cm. The three remaining skin areas served as controls A representative piece of the normal and treated skin areas was taken for microscopic study 2 days after the last treatments. The methylcholanthrene treated skin showed the same gross changes described in Experiment 2 A 5 per cent virus filtrate (W R. 1-30) was then rubbed into one area of each sort after scarification and into another after dilution of the filtrate to 0 1 per cent. The inoculations were made immediately after the biopsies

Table II shows the results of the experiment On the *12th day* after virus inoculation the benzene and methylcholanthrene-treated patches inoculated with 0 1 per cent filtrate showed discrete papillomas in three of the four test rabbits No growths could be seen on the normal or x rayed skin areas at this time All of the areas inoculated with 5 per cent filtrate showed papillomas the growths on the methylcholanthrene-treated areas being large semiconfluent masses in contrast to the few small discrete growths just appearing on the normal and x rayed patches Many more discrete papillomas were present on the benzene treated areas than on the normal skin, but they were fewer in number and smaller than on the skin areas which had received methylcholanthrene By the *35th day* the papillomas on the benzene and methylcholanthrene-treated areas inoculated with the dilute filtrate had developed into confluent and semiconfluent papillomatous masses, whereas the growths on the normal and x rayed skin areas produced with the same inoculum were still small and discrete though increasing in number The skin areas inoculated with 5 per cent filtrate all showed confluent papillomas, but those on the normal and x rayed patches were low mounds, up to 0 6 cm. high whereas on the benzene treated areas they were jagged peaks up to 1 0 cm. high and on the skin treated with methylcholanthrene they were up to 1 6 cm high The last mentioned growths were deeply pigmented, while the others were pink or merely streaked with gray Microscopic sections of the growths showed them all to be ordinary papillomas

To extend the findings another experiment was done This time the skin areas were treated with ultraviolet light, methylcholanthrene, and a mixture of turpentine and acetone.

Experiment 4—A mixture of turpentine and acetone in equal parts,—which had proved non-carcinogenic (8),—was applied to three clipped areas on the abdomens of four domestic rabbits five times at 2 day intervals and 0.3 per cent methylcholanthrene in benzene in like manner to another three Three other areas were exposed to a quartz mercury vapor lamp[3] for 40 minutes at a distance of 25 cm. 48 hours before virus inoculation. This caused a marked erythema Three untreated skin areas served as controls The turpentine and acetone-treated skin became acutely inflamed, thickened and hyperkeratotic, as greatly changed in the gross as the methylcholanthrene-treated skin A virus filtrate (W.R. 1 70) in concentrations of 0 1 per cent and 5 per cent was rubbed into two rows of the areas after scarification

The results are shown in Table III. It will be seen that the papillomas on the methylcholanthrene- and turpentine and acetone-treated areas arose earlier and in

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[3] Alpine sun lamp, Hanovia Chemical and Manufacturing Company

TABLE II

Susceptibility of Normal Skin and Skin Treated with X-Rays, Benzene, and Methylcholanthrene

)ilution of rus filtrate V.R. 1 30	Skin treatment before virus inoculation*	Pathogenicity tests										
		12th day				18th day				3th day		
st rabbits		a	b	c	d	a	b	c	d	a	b	c
per cent												
0 1	Normal skin	0	0	0	0	±	±	+	±	++	+±	++
	X rays	0	0	0	0	±	+	0	0	+±	++±	+±
	Benzene	+	+	0	+	+±	++	++	++	+++±	+++±	++-
	Methylcholanthrene	±	+	0	±	+±	++	++±	+±	+++±	+++±	+++
5	Normal skin	±	+	±	+	++	+++	++±	++±	++++	++++	+++
	X rays	±	+	±	±	++	+++	+++	+±	+++±	++++	+++
	Benzene	++	++±	++	++	+++±	++++	+++±	+++±	++++	++++	+++
	Methylcholanthrene	+++	+++±	+++	+++±	++++	++++	++++	++++	++++	++++	+++

* Benzene applied to skin areas three times per week for 2 weeks before virus inoculation
0 3 per cent methylcholanthrene in benzene applied three times per week for 2 weeks before virus inocul
in
X-ray irradiation, 1500 r
Virus inoculated 2 days after last treatments

TABLE III

Susceptibility of Normal Skin and Skin Treated with Ultraviolet Light, Turpentine and Acetone, and Methylcholanthrene

ilution f virus ltrate .R 1 70	Skin treatment before virus inoculation*	Pathogenicity tests											
		11th day				18th day				35th day			
t rabbits		a	b	c	d	a	b	c	d	a	b	c	d
er cent													
0 1	Normal skin	0	0	0	0	±	+±	+	+±	++	+++±	++±	+++±
	Ultraviolet light	0	0	0	0	+±	+±	+	+	+++	+++±	++±	+++
	Turpentine acetone	±	+	0	±	+++	+++±	++	+++	++++	++++	++++	++++
	Methylcholanthrene	0	±	+	0	+++	+++	+++	++±	++++	++++	++++	++++
5	Normal skin	0	++	+	+	+++	+++±	+++	+++±	++++	++++	++++	++++
	Ultraviolet light	+	++±	++	++	+++±	++++	+++	++++	++++	++++	++++	++++
	Turpentine-acetone	+±	+++	++±	+++	++++	++++	++++	++++	++++	++++	++++	++++
	Methylcholanthrene	+±	++±	+++±	+++	+++±	++++	++++	++++	++++	++++	++++	++++

* Ultraviolet light, 40 minutes irradiation from a carbon lamp 24 hours before virus inoculation
Turpentine and acetone in equal parts applied to skin five times at 2 day intervals
0.3 per cent methylcholanthrene in benzene applied to skin five times at 2 day intervals

greater numbers than on those which had been normal There was no significant difference between the growths on the treated areas and they all soon became large, confluent papillomatous masses The papillomas arising on the areas which had been exposed to ultraviolet light were like those on the normal skin in number and size

The results of Experiments 2, 3, and 4 (Tables I, II, and III) show that a variety of agents some carcinogenic, others not, will render the skin abnormally susceptible to virus infection A mixture of turpentine and acetone was as effective in this respect as was tar or methylcholanthrene These findings have been confirmed in many subsequent tests in which the procedures have been utilized for various purposes Mere acute inflammation produced by ultraviolet light did not render the skin more susceptible, nor did the Roentgen rays

Optimal Preparation of the Skin for Virus Infection

Steps were now taken to determine the number of applications of methyl cholanthrene or of turpentine and acetone which renders the skin most susceptible to the papilloma virus.

Experiment 5 —0 3 per cent methylcholanthrene in benzene was painted onto a skin area of each of four domestic rabbits at 2 day intervals for a total of six times, and another comparable skin area was simultaneously treated with a mixture of turpentine and acetone Two other skin areas of the same animals were treated with the agents three times at 2 day intervals, while another two areas received but a single application The schedule was so arranged that the last application was made 24 hours prior to virus inoculation Two skin areas on each rabbit were left untreated. As usual the situation of the treated areas was varied. A single application of methylcholanthrene caused only a reddening of the skin after 24 hours After three applications however the skin was acutely inflamed thickened and covered with a branny scurf Six treatments caused even greater changes with marked thickening and much scurf, some of which could be flaked away Turpentine and acetone caused similar changes, but the skin was slightly less thickened, although more inflamed Biopsy specimens were taken from all of the areas Immediately afterward the areas were scarified and a 0.5 per cent virus extract (W.R. 1 28) was rubbed into them.

The results are summarized in Table IV It will be seen that a single application of methylcholanthrene or turpentine and acetone did not alter the susceptibility of the skin to papilloma virus infection, the incubation period and the number and size of growths were about the same on the normal and treated skin areas Those treated three times, however, showed numerous papillomas before any could be seen on the normal areas and they had become large, confluent masses, up to 1 6 cm high at a time when growths on the normal skin were still discrete or semiconfluent and no more than 0 4 cm high at most. Six applications of the agents rendered the skin only slightly more susceptible to virus infection than did three applications, in spite of the fact that it appeared much more changed, and histologically was really so, as will be shown further on

Six applications of methylcholanthrene or turpentine and acetone at 2 day intervals are about the maximum that the skin of the rabbit's abdomen will bear without becoming macerated[3] It will withstand many applications of methylcholanthrene, however, if applied at 3 to 4 day intervals Further experiments have shown that many treatments with these agents fail to render the epidermis more susceptible than do three to six applications at 2 day intervals

TABLE IV

Susceptibility of Skin Prepared for Various Lengths of Time

Agent applied to skin before virus inoculation*	No of applications	Pathogenicity tests‡											
		12th day				16th day				24th day			
Test rabbits		a	b	c	d	a	b	c	d	a	b	c	d
Normal skin		0	0	0	0	+	+±	±	++	+++	+++	++	+++
Turpentine acetone	One	0	0	0	0	++	+	+	+	+++±	++±	++	+++
	Three	+±	+±	+±	++	+++	+++	+++	+++	++++	++++	++++	++++
	Six	+	+++	+++	+	+++	+++±	+++±	+++	++++	++++	++++	++++
Methylcholanthrene	One	0	0	0	0	+	±	±	+±	+++	++	++	+++
	Three	+±	+±	+	++	++	+++	++±	+++	++++	++++	++++	++++
	Six	+±	++	++±	++	+++	+++	+++±	+++	++++	++++	++++	++++

* Turpentine and acetone in equal parts

Methylcholanthrene, 0 3 per cent in benzene

‡ Virus extract, W R 1-28, 0 5 per cent, rubbed into scarified skin 24 hours after preparation

Duration of the Abnormal Susceptibility

Does the increased susceptibility of methylcholanthrene-treated skin to papilloma virus infection persist when applications of methylcholanthrene are discontinued? To answer this question skin areas were treated with methylcholanthrene in the next experiment and then inoculated with papilloma virus at various intervals from 1 day to 1 month after completion of the treatments

Experiment 6—Five areas on each of five rabbits were painted with 0.3 per cent methylcholanthrene in benzene at intervals of 2 days for a total of four times The treatment of each skin area was begun on a different date so that from 24 hours to 4

[3] The agents mentioned induce a profuse growth of hair It has been found that removal of this with clippers prior to each application provides a better prepared epidermis which can be readily scarified

weeks had elapsed on the day when they were all inoculated with 0 1 per cent virus filtrate (W.R E) Just prior to inoculation biopsies were made as usual.

Table V shows the results of the Experiment. As noted in the preceding tests, papillomas appeared earlier and in greater number on the methylcholanthrene-treated skin inoculated 24 hours after completion of the treatments than on scarified normal skin The areas inoculated with virus 1 week afterwards showed slightly fewer papillomas in four of the five test rabbits but in one instance there was no difference The skin inoculated 3 weeks after discontinuation of the methylcholanthrene applications showed more growths than the normal skin in two instances only, and the patches inoculated 4 weeks after the applications had been stopped yielded the same results as the normal

TABLE V

Duration of the Increased Susceptibility of Methylcholanthrene Treated Skin

Time from skin treatment* to virus inoculation‡	Pathogenicity tests														
	14th day					16th day					21st day				
Test rabbits	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
1 day	+	++	+++	+++	+±	++	++	+++	+++	++±	+++	++++	++++	++++	+++±
1 wk.	±	+±	+±	++±	+±	++	+±	++	+++	++	+++	+++	+++±	++++	+++
2 wks.	±	+	0	++	+	++	+	±	++±	+±	++	+±	+±	+++	++±
3 wks.	0	0	0	+±	±	±	±	±	++	+±	+	+±	+	++	++±
4 wks.	0	0	0	0	0	+	±	±	+±	±	+	+	+±	++	+±
Normal skin	0	0	0	0	0	+	±	±	+	±	++	+	+±	++	+

* 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals.
‡ Virus filtrate, W.R. E, 1 1000

skin It is thus apparent that the increased reactivity of methylcholanthrene treated skin to papilloma virus infection is transitory and is lost within 2 to 4 weeks after the treatments are stopped

Comparative Titrations of the Virus on Normal and Altered Skin

The increased number, shortened incubation period, and the rapidity with which the papillomas enlarged when virus was inoculated into properly prepared skin pointed to a markedly increased effectiveness of the virus. Experiments were next undertaken to learn whether it would be infectious in dilutions which yield no growths under ordinary circumstances The virus is rarely infectious on the scarified normal skin of domestic rabbits in dilutions beyond 1 100,000 (in terms of weight of papilloma tissue extracted) (6)

Experiment 7 —Six squares were clipped on one side of the belly of four rabbits, and painted with a mixture of turpentine and acetone five times at 2 day intervals They

showed the usual alterations Six corresponding squares on the other side were not treated All were scarified and a papilloma virus extract (W R 1-28) in dilutions of from 1 10,000 to 1 10,000,000 was rubbed into corresponding normal and treated squares The inoculations were made 24 hours after the last of the turpentine acetone applications

It will be seen (Table VI) that by the 16th day after inoculation all of the treated areas which had received the 1 50,000 dilution of virus showed papillomas and the 1 100,000 had also caused them in one animal No growths whatever had appeared at this time on the control squares Later the control areas showed papillomas in every case as result of the 1 50,000 dilution, and the 1 100,000 dilution also caused them in the more susceptible animals But

TABLE VI

Titration of a Virus Preparation in Normal and in Turpentine and Acetone Treated Skin

Dilution of virus extract W R 1 28	Pathogenicity tests															
	16th day								42nd day							
	Normal skin				Turpentine and acetone treated skin*				Normal skin				Turpentine and acetone treated skin*			
Test rabbits	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
1 10 000	0	0	0	0	+	+	+±	+±	+++±	+++±	+++	++±	++++	++++	++++	++++
1 50 000	0	0	0	0	±	±	±	±	+	+++	+	±	+++	+++	+++	++±
1 100 000	0	0	0	0	0	±	0	0	±	++±	±	0	+++	+++	++	+
1 500 000	0	0	0	0	0	0	0	0	0	0	0	0	+	++	++	±
1 1 000 000	0	0	0	0	0	0	0	0	0	0	0	0	±	+	±	0
1 10 000 000	0	0	0	0	0	0	0	0	0	0	0	0	0	±	0	0

* Turpentine and acetone in equal parts applied three times per week for a total of five times before virus inoculation

the virus had proved greatly more effective on the treated areas At 1 100,000 it regularly produced papillomas, as did the 1 500,000 dilution, while in three of the four animals the 1 1,000,000 dilution proved effective, and in one individual the virus extract yielded papillomas when diluted 10,000,000 times

The experiment shows that not only was the number of papillomas increased and the incubation period shortened by the use of treated skin but the titer of the virus was much stepped up—from 10- to 100-fold Another, similar experiment, using methylcholanthrene-treated skin and another virus extract, yielded practically identical results In a subsequent paper many instances will be given, incidentally to other work, of the enhanced susceptibility of treated skin

Effect of Skin Alterations before and after Virus Inoculation

It seemed possible that methylcholanthrene or turpentine and acetone applications to the epidermis after virus inoculation might have the same

effect as preliminary treatment. An experiment was done to test the possibility

Experiment 8 —Nine areas in three parallel rows were clipped on the abdomens of four rabbits Those of one row were painted with 0.3 per cent methylcholanthrene in benzene at 2 day intervals for a total of four times, with result in the usual changes The other six patches were not treated Two days after the last treatment all were scarified and inoculated with a virus extract (W R. 2 95) in dilutions of 1 50 000,

TABLE VII

Infectivity of Virus in Skin Treated with Methylcholanthrene before and after Inoculation

Skin treatment	Dilution of virus extract W.R 2-95 used for inoculation	Pathogenicity tests											
		18th day				28th day				35th day			
Test rabbits		a	b	c	d	a	b	c	d	a	b	c	d
Methylcholanthrene applied to skin four times *before* inoculation	1 50 000	+±	++	+±	++	+++	++++	+++	+++±	+++±	++++	+++	+++±
	1:500 000	±	+	±	+	+±	++	+	++	++	++	+	++
	1 5 000 000	0	±	0	0	+	+	±	±	+	+±	±	±
Methylcholanthrene applied to skin four times *after* virus inoculation‡	1 50 000	±	0	0	±	±	+	0	±	±	+	0	±
	1 500 000	0	0	0	0	0	0	0	0	0	0	0	0
	1 5 000 000	0	0	0	0	0	0	0	0	0	0	0	0
Normal skin	1 50 000	±	0	0	0	++	+	±	0	++	+	±	0
	1 500 000	0	0	0	0	±	0	0	0	±	0	0	0
	1 5 000 000	0	0	0	0	0	0	0	0	0	0	0	0

* 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals before virus inoculation

‡ 0.3 per cent methylcholanthrene in benzene applied to skin four times at 2 day intervals beginning 5 days after virus inoculation

1 500,000, and 1 5 000,000 One area of each row received the same dilution of virus Five days later, when the skin areas had almost healed, one of the rows of patches which had not been treated prior to inoculation was painted with 0.3 per cent methyl cholanthrene in benzene and the applications were kept up at 2 day intervals for a total of four times, again inducing the familiar changes To the other rows of patches nothing further was done.

The results of the experiment are set down in Table VII On the 18th day after inoculation the virus diluted 1 500,000 had caused papillomas on all of the patches treated with methylcholanthrene prior to inoculation and so too had a dilution of 1 5,000,000 in one rabbit. The control patches and those treated with methylcholanthrene after inoculation showed at this time no

papillomas where virus had been inoculated in dilutions above 1 50,000, and they were only just appearing where this latter had been put By the 35th day (after which no growths appeared) the 1 5,000,000 dilution of the virus had produced papillomas on all of the skin areas treated before inoculation, but had failed to cause growths on untreated areas or on those that were treated with methylcholanthrene after virus inoculation 1 50,000 caused large, confluent or semiconfluent papillomatous masses on all of the areas prepared before inoculation, but it produced only a few discrete growths on the other areas

Manifestly, methylcholanthrene treatment of the skin after virus inoculation did not enhance the effects of the virus A similar experiment, using a mixture of turpentine and acetone instead of methylcholanthrene, yielded similar results except that fewer growths arose on the skin treated with the turpentine and acetone after scarification than on the untreated skin

Results of Altering the Skin of Cottontail Rabbits

The work was now extended to cottontail rabbits, the natural hosts of the papilloma virus, to learn whether their skin can be rendered more susceptible to the virus [4]

Several experiments of the sort already described were frustrated by skin injury, maceration and bacterial infection occurring when two to four applications of methylcholanthrene or the mixture of turpentine and acetone were applied at 2 day intervals, —procedures well tolerated in the domestic rabbit However, when the intervals were lengthened to 4 days the skin underwent the same gross changes as in these latter animals But unfortunately it also became notably susceptible to bacterial infection and scarification frequently resulted in broad abscesses To avoid these, resort was had to intradermal inoculations of the virus These gave irregular results, yet by and large the several experiments gave clear indications that methylcholanthrene, benzene, and a mixture of turpentine and acetone, all render the epidermis of the cottontail rabbit unusually susceptible to the papilloma virus

Nature of the Skin Changes

The agents that enhanced the susceptibility of the skin for the papilloma virus all caused marked alterations of the same general sort In the gross they consisted of thickening, scurfing, increased pigmentation, and more or less

[4] The papilloma virus ordinarily titers slightly higher in susceptible cottontails than in the domestic species Table VIII shows the results of rubbing graded dilutions of a virus extract (W R 1-28) into scarified skin areas of six normal domestic rabbits and six cottontail rabbits One cottontail proved immune to the virus and hence was not included in the table It will be seen that the virus extract produced growths in all of the cottontail rabbits in dilutions up to 1 800,000, whereas this last caused none in any of the domestic rabbits, and in only two of the six did a dilution of 1 400,000 cause any

acute inflammation The extent of the changes, however, varied with the agent used

The microscopic findings can be briefly summarized —

The *normal skin* of the abdomen of the domestic rabbit has a thick connective tissue stroma and a mere skim of superficial epidermis (Fig 1), consisting of a Malpighian layer one to three cells thick which keratinizes abruptly without differentiation The hair follicles do not go deep and the sebaceous glands are small and inconspicuous. Three to four applications of *tar* to the skin were found to cause striking changes (Fig 2) The Malphighian layer became greatly thickened and the cells appeared larger than normal and showed many mitoses. They differentiated gradually forming a granular layer and a thick stratum of keratinization The hair follicles became greatly distended with keratin in many instances Numerous sebaceous glands appeared and the dermis showed an acute inflammatory reaction with edema small hemorrhages, and cellular infiltration All these changes have been often described before A single application of *methylcholanthrene* caused a mild inflammatory reaction in the dermis and the basal cells of the epidermis appeared to be slightly larger than normal but were not increased in number After three or four applications the epidermis was markedly hyperplastic and the changes were similar to those seen in the tarred skin, but lesser in degree (Fig 8) After six applications there was even greater thickening and irregularity of the epidermis and portions of the skin were necrotic. When the treatments were discontinued the skin gradually reverted to the normal. Within 1 week after the skin had received four applications of methylcholanthrene, regression was evident. The epidermis was still thicker than normal and exhibited a graded differentiation but the cells showed far fewer mitoses than 24 hours after the treatments and were smaller and less abnormal Within 2 weeks involution was advanced, and after 3 weeks the epidermis had returned to normal save for a slight hyperkeratosis The hair follicles appeared normal now except at the bases where there was still some thickening of the epithelial layer and the cells showed numerous mitoses and were slightly larger than normal. A few polymorphonuclear leucocytes could still be seen in the connective tissue. After 4 weeks the skin seemed wholly normal. *Benzpyrene* caused changes similar to those induced by methylcholanthrene except that the sebaceous glands were more hyperplastic and occasionally cystic.

The non-carcinogenic agents which were effective in increasing the susceptibility of the skin for the virus brought about much the same histological changes. *Turpentine and acetone* caused less hyperplasia of the epidermis than methylcholanthrene (Fig 3) but a more acute inflammatory reaction in the subcutaneous tissue The *benzene-treated skin* was less hyperplastic than that altered by methylcholanthrene but the general changes were similar Occasionally there were small patches of epidermal necrosis The sebaceous glands were markedly hyperplastic and showed differentiation

Ultraviolet light caused an acute inflammatory reaction 48 hours after irradiation, but the epidermis was still as thin as usual at this time and sometimes showed small areas of necrosis *X-ray irradiation* elicited no significant changes in the skin under the conditions employed (Experiment 3) These agents failed to alter the susceptibility of the skin for the virus as Experiments 3 and 4 showed

The microscopic changes elicited in cottontail rabbit skin by the carcinogenic and non-carcinogenic agents were at least as marked as those seen in the domestic rabbit and usually more so. Two to three applications of methylcholanthrene, benzene, or turpentine and acetone at intervals of 4 days changed the epidermis of the wild rabbit from a thin, delicate epithelium one to two cells thick, to a greatly thickened and irregular, differentiating sheet, with distended, hyperplastic hair follicles and numerous sebaceous glands. Further applications usually rendered the epidermis necrotic.

The findings plainly showed that the various agents which enhanced the susceptibility of rabbit skin all caused the epidermis to become hyperplastic and to proliferate actively. The agents which proved ineffective,—ultraviolet light and x-ray,—did not do so, although the former produced an acute inflammatory reaction in the skin.

The Skin Changes Induced by Scarification

Several authors have described the early stages in virus-induced papillomatosis (9) but no one has inquired into the changes which follow immediately upon scarification of the skin and virus inunction. It seemed probable that a knowledge of these changes would aid toward an understanding of the reasons for the increased susceptibility of altered skin, and consequently a study of them was undertaken. Papilloma virus was rubbed into some scarified areas and a solution of minute graphite particles (hydrokollag (10)) into others, to learn the fate of particulate matter as such.

Experiment 9.—Nine skin areas on the abdomens of three domestic rabbits were painted with 0.3 per cent methylcholanthrene in benzene at 2 day intervals for a total of four times. 24 hours after the last treatment a representative piece of the changed skin was removed from each animal. All of the areas were then scarified with sand paper to the usual extent, that is to say, until there was oozing of serum, sometimes blood-tinged, and a 5 per cent papilloma virus extract (W.R.D.) was rubbed into three of the patches, a dilute suspension of hydrokollag in saline into three others, while the remaining three areas were left as such. In addition nine untreated areas on each of three other rabbits were similarly scarified and the virus and the hydrokollag suspension were rubbed into them. Slices of skin were taken from all of the various areas 5 hours after scarification, and additional pieces were procured after 1, 2, 4, 6, 8, 11, and 14 days. They were fixed in acid Zenker's and stained with eosin and methylene blue. Duplicate sections from the areas receiving hydrokollag were stained with Lichtgrün as well.

It was found that scarification of the *normal skin* to the extent ordinarily employed for virus inoculation removed practically all of the epithelial covering and some of the superficial connective tissue as well, cutting across the hair follicle shafts near the surface. Only occasional small islands of surface epidermis were left. After 24 hours the connective tissue was edematous and showed a few polymorphonuclear leucocytes and a thin scab had formed on the surface, but as yet no epithelial repair had taken place. The scab had become much thicker after 24 hours, owing not only to the ac

cumulation of dried exudate but mostly to necrosis of the superficial connective tissue layer with incorporation of the dead material in the scab After 48 hours epithelial regeneration was for the first time plainly evident. It took origin almost entirely from the cells of the hair follicle shafts, the epithelial cells extending laterally between scab and living connective tissue and sometimes into crevices in the latter The first extension seemed to be mostly migration though occasional mitoses could be seen The newly formed cells showed many mitoses later After 2 to 4 days they had multiplied and spread laterally to such extent as to form umbrella like expanses with the hair follicles as the staffs of the umbrellas At this time the epidermis had not nearly covered all of the denuded surface. The connective tissue was still edematous and showed some round cells and polymorphonuclear leucocytes but the scab had begun to separate here and there After 4 to 8 days the surface was entirely covered with a layer of hyperplastic epithelium (Fig 5) three to six cells thick which had differentiated into granular and keratinized layers. The picture was markedly different from the normal (Fig 1) The hair follicle shafts had thickened only slightly except next the orifices where the epithelium was hyperplastic. There was no evident stimulation of the sebaceous glands. At this time the surface scab had only just come away and remnants of it could be seen Later the hyperplastic epidermis slowly took on a normal appearance and by the 14th day it had nearly done so though here and there it was still slightly thickened and hyperkeratotic.

The skin areas inoculated with papilloma virus showed similar general changes during the first days, and with the inoculum employed —which gave rise to growths relatively late,—changes referable to the virus were not discerned until 6 to 8 days had elapsed Then the characteristic alterations of early papillomatosis (9) could be made out here and there By the 11th to the 14th day there were discrete characteristic growths. The microscope showed most of them to have arisen from the basal layers of the new hyperplastic epidermis which now covered the surface between the hair follicles.

The areas into which hydrokollag had been rubbed showed the black particles in close contact with the abraded surface immediately after the inunction and in direct contact with the epithelium of the hair follicles that had been cut across. But as the scab thickened owing to the necrosis of the superficial tissue with incorporation in it of the dead layer almost all of the hydrokollag became incorporated too, only traces remaining in crevices here and there where it might come in contact later with the regenerating epithelial cells. Careful search was made for phagocytosis of it by these elements but none could be found, and when the newly formed epidermis keratinized, by the 4th to the 8th day after scarification, it was all cast off

These findings show that the scarification of normal skin as ordinarily done removes nearly all of the surface epidermal cells and entails a loss of most of the virus (if one can judge from what happens to hydrokollag) in the scab which forms, the result being that the chance for it to reach susceptible cells is greatly reduced Almost the only spots at which virus is directly brought into contact with epithelium are where the hair follicles have been cut across and even here the epithelium commonly becomes implicated in the later

necrosis and is lost Papillomas derived from the hair follicle epithelium are relatively infrequent Instead they usually appear on the surface between the hair follicles and one must suppose they principally arise from young, regenerating cells which have extended laterally from the follicles to cover the denuded surface

Scarification of the *methylcholanthrene-treated skin* (Fig 8) was done to the same extent as with normal skin, that is to say, until the surface of the patch seemed everywhere abraded and serum oozed out Though the surface epithelium was markedly hyperplastic, the microscope showed that practically all of it had been removed as in the case of the normal skin, exposing the fibrous corium and the hair follicle shafts (Fig 9) These latter were hyperplastic The connective tissue became edematous as usual, polymorphonuclear leucocytes wandered into it, and scabbing took place with increase in the thickness of the scab by superficial necrosis (Fig 10) But epithelial regeneration was far more rapid than under ordinary circumstances Within 24 hours after scarification it was already far advanced (Fig 10) and within 48 hours the entire abraded surface was covered (Fig 11) Migration began by lateral extension from the hair follicles, and was attended by such active multiplication that very soon large, thick, umbrella-like structures with the follicles as the shafts of the umbrellas had formed beneath the surface scab (Fig 12) They consisted of great numbers of epidermal cells, many of them in mitotic division, and as these spread along the surface beneath the scab they frequently invaded irregularly the crevices in the fibrous corium The sheet of hyperplastic, actively regenerating epidermis that rapidly formed was much thicker than that produced by the regeneration of ordinary epidermis (Fig 5) and consisted of a shallow keratinized layer and a Malpighian layer ten to fifteen cells deep, showing numerous mitoses Irregular papillae extended down into the connective tissue The hair follicles were distended with keratinized epithelium and the epithelial lining was thickened There appeared to be a notable increase in the number of sebaceous glands and they were rendered prominent not only by hyperplasia but by retention of secretion which caused many of them to be actually cystic It seems probable that the hyperplastic surface epithelium had become so crowded through active proliferation as to prevent escape of their contents Four to 6 days after scarification the epidermis was more orderly, differentiating into wide granular and keratinized layers, and as keratinization progressed the scab came away Involution took place rapidly and by the 14th day the epithelium had returned practically to normal save for irregularities of basal contour and local thickenings where the layer of living cells was still three to four cells deep The hyperkeratosis was now in general less than that where the normal skin had been scarified 14 days previously While involution was going on the sebaceous glands at first became still more cystic, some of them rounding out into small spheres but later the distention of them disappeared and they again became inconspicuous

The methylcholanthrene treated areas which were inoculated with virus after scarification underwent similar changes The effects of the virus were evident in them earlier than when scarified normal skin had received the same inoculum, the characteristic cytological changes indicative of the beginning of papillomatosis being perceptible in one rabbit 4 days after inoculation and on the 6th day in others The fate of the

hydrokollag was the same as when rubbed into scarified normal skin. Practically all of it was caught in the scab and came away when this did

From these findings it is plain that the changes which render skin especially susceptible to the virus provide to the latter young, actively proliferating epidermal cells in unusually great number and at a much earlier stage in events than when normal skin has been scarified Where the hair follicles have been cut across by the sandpaper many more cells are exposed to direct infection with the virus, and where it persists under the scab the regenerating epithelium soon reaches it.

Intradermal Inoculation of Virus into Prepared Rabbit Skin

Despite the defects of inoculation into scarified areas as just disclosed, it has proved the most certain method to the present for titrating the papilloma virus. Intradermal injection of active virus into normal skin does not always result in growths, and the incubation period is regularly longer than in scarified skin (4)[5] It has seemed worth while nevertheless to determine the effects of the intradermal inoculation of virus in skin altered by methylcholanthrene Accordingly papilloma virus was so inoculated and was also rubbed into scarified normal and methylcholanthrene treated skin areas at comparable situations in the same animals. Incidentally, to enlarge the general findings, a mixture of methylcholanthrene and Scharlach R was applied to some areas, and the dye was injected intradermally into other areas that had also been treated with methylcholanthrene prior to virus inoculation Scharlach R is known to cause a profuse epidermal proliferation and sometimes temporary downgrowths simulating early carcinomatosis (11)

Experiment 10 —Four rectangular areas were clipped on each side of the abdomens of four rabbits. One on each side was painted with 0.3 per cent methylcholanthrene in benzene, another with a saturated solution of Scharlach R in benzene containing 0.3 per cent methylcholanthrene, while a third was painted with 0.3 per cent methyl cholanthrene in benzene and 0 1 to 0.2 cc. of a saturated solution of Scharlach R in olive oil was injected intradermally at several places. The treatments were repeated four times at 2 day intervals The fourth area on each side served as control Two days after the last treatments pieces were taken from each area of two of the rabbits for microscopic study The mixture of methylcholanthrene and Scharlach R caused much greater skin alterations than did methylcholanthrene alone. The thickened skin was rendered far more rugose and became so redundant as to be thrown into folds. Microscopically, the changes resembled in many ways those seen in the tarred epidermis, already described, but the thickening of the surface layer of epithelium

[5] This is also true of tattoo inoculations.

virus, herpes virus, and virus III is found in the young cells filling in the defects consequent on scarification (13).

The experiments here reported to learn the effects of the scarification preliminary to virus inoculation and the stages in its repair have disclosed that most of the surface epithelium is scraped away and that practically all of what is left, and the superficial connective tissue as well, is destroyed in the scab which forms within the next 2 days. As already pointed out, the virus rubbed into the raw surface must also be lost in the scabbing and but little can remain here or there underneath the dead material to infect the young, regenerating epidermal cells which extend from the hair follicles. It is possible that some direct infection of the preexisting hair follicle cells may occur at the time when the shafts of the follicles are cut across by the sandpaper and the virus is rubbed in, but this seems unlikely because the scabbing involves them too. Indeed, microscopically one seldom sees papillomas beginning at the hair follicles, these ordinarily arising from the reconstituted surface sheet of epithelium which lies between them.

The various agents which render the skin more susceptible to infection with the virus all effect changes which should bring young, actively regenerating cells into association with the virus in much greater number than ordinary and at a much earlier time. They also increase the local vascularity, and to this circumstance as well as to a much more abundant initial cell infection the shortened incubation period and subsequent rapid enlargement of the papillomas can be laid. Olitsky and Schlesinger (14) have recently shown that local edema produced by the subcutaneous injection of hypertonic solutions prior to inoculation of the skin with herpes virus greatly increases the effectiveness of the latter, and Sprunt (15) has brought evidence to show that susceptibility to infection with vaccine virus is influenced by the number of cells exposed to the latter. But the increased susceptibility of the altered skin for papilloma virus may be due to more than the provision of a richer vascularization and of an increased number of susceptible cells for infection. The individual young cells of an epidermis regenerating after alteration by the preparatives of the present work may be especially susceptible to the virus.

Under the ordinary circumstances of scarification and virus inunction infection takes place at scattered points with secondary coalescence, the result being separate more or less broad-based papillomas or confluent papillomatous expanses. The scattered character of the initial infection explains why confluent masses usually have craggy peaks with clefts between that frequently extend down to the skin level, and why on cross-section local differences can be perceived, which are expressions of the proliferation of differing infected cell families (16). Only occasionally does infection by scarification result in growths so compacted as to suggest that a broadcast, primary infection has

taken place Naturally occurring papillomas in cottontails are not infrequently compacted, however, having the form of solid discs or "onions," presumably because they are the outcome of primary punctate infection with expansile enlargement. Growths of similar sort can be produced experimentally in both cottontails and domestic rabbits by intradermal or tattoo inoculation

Bryan and Beard (17) have lately laid stress on the length of the incubation period (time elapsing after inoculation before papillomas are visible in the gross) as a reliable indicator of the amount of active virus present in the inoculum There can, of course, be no doubt that the greater the number of virus entities distributed upon a scarified area the greater the chance will be for cells to become infected, up to a certain maximum, other things being equal and,—since most papillomas are consequent on multiple cell infection,—the sooner should the papillomas appear But if the skin is abnormal other factors enter into the matter, as the present work shows, notably the regenerative activities of the epidermis and the local vascular state The rate of appearance of papillomas is conditioned not only by virus quantity and pathogenicity but by the state of the tissue acted upon

The experiments here reported provide a practicable method to render papilloma virus many times more effective on experimental inoculation Papilloma extracts that do not elicit growths in normal skin in dilutions beyond 1 100,000 produce growths in methylcholanthrene- or turpentine and acetone-treated skin in dilutions of 1 1,000,000 or 1 10,000,000, while furthermore the *incubation period of the papillomas in the altered skin is considerably shorter* than in normal skin It has been calculated that about 94,000,000 papilloma protein molecules are present in the dilution giving the 50 per cent point (18) The findings of the present experiments suggest that this figure mostly expresses the difficulty of bringing virus into association with susceptible cells By the means described in the present work the number of entities in the inoculum requisite to infection can be reduced considerably, although under the circumstances of virus inunction into scarified skin most of the virus is still lost by the way To gauge the effectiveness of individual papilloma virus entities one would have to be certain that these reached the appropriate cells and that the latter were in a state to be infected Needless to say, these considerations apply to other viruses as well.

Experiments carried out with Dr John G Kidd have shown that skin preparation by the methods here described is useful in demonstrating papilloma virus in materials which fail to give rise to growths when inoculated in the ordinary way Extracts of domestic rabbit papillomas which are non pathogenic when so tested, in which, that is to say, the virus appears to be "masked," produce growths in most instances when inoculated into methylcholanthrene-

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Bryan and Beard (17) have lately laid stress on the length of the incubation period (time elapsing after inoculation before papillomas are visible in the gross) as a reliable indicator of the amount of active virus present in the inoculum. There can, of course, be no doubt that the greater the number of virus entities distributed upon a scarified area the greater the chance will be for cells to become infected, up to a certain maximum, other things being equal and,—since most papillomas are consequent on multiple cell infection,—the sooner should the papillomas appear But if the skin is abnormal other factors enter into the matter, as the present work shows, notably the regenerative activities of the epidermis and the local vascular state The rate of appearance of papillomas is conditioned not only by virus quantity and pathogenicity but by the state of the tissue acted upon

The experiments here reported provide a practicable method to render papilloma virus many times more effective on experimental inoculation Papilloma extracts that do not elicit growths in normal skin in dilutions beyond 1 100,000 produce growths in methylcholanthrene- or turpentine and acetone-treated skin in dilutions of 1 1 000,000 or 1 10,000,000, while furthermore the incubation period of the papillomas in the altered skin is considerably shorter than in normal skin It has been calculated that about 94,000,000 papilloma protein molecules are present in the dilution giving the 50 per cent point (18) The findings of the present experiments suggest that this figure mostly expresses the difficulty of bringing virus into association with susceptible cells By the means described in the present work the number of entities in the inoculum requisite to infection can be reduced considerably, although under the circumstances of virus inunction into scarified skin most of the virus is still lost by the way To gauge the effectiveness of individual papilloma virus entities one would have to be certain that these reached the appropriate cells and that the latter were in a state to be infected Needless to say, these considerations apply to other viruses as well.

Experiments carried out with Dr John G Kidd have shown that skin preparation by the methods here described is useful in demonstrating papilloma virus in materials which fail to give rise to growths when inoculated in the ordinary way Extracts of domestic rabbit papillomas which are non pathogenic when so tested, in which, that is to say, the virus appears to be "masked," produce growths in most instances when inoculated into methylcholanthrene-

or turpentine and acetone-treated skin These findings will be reported in detail in a forthcoming paper

SUMMARY

Rabbit skin can be rendered abnormally susceptible to papilloma virus infection by preliminary treatments with a variety of agents The most effective agents thus far found are 0 3 per cent methylcholanthrene in benzene and a mixture in equal parts of turpentine and acetone, applied four or five times at 2 day intervals When virus is inoculated into skin altered by these agents, either intradermally or by inunction after scarification, papillomas appear earlier and in greater number than on normal skin, and much higher dilutions give rise to growths The method provides a means of detecting amounts of virus which cause no papillomas upon inoculation into normal skin

Papilloma virus material which is rubbed into scarified normal or hyperplastic skin is largely lost in the scabbing which ensues, and nearly all of it fails to reach susceptible cells The preparatory agents which increase the effectiveness of the virus bring about marked epidermal hyperplasia, and the hyperplastic tissue regenerates with greater rapidity when scarified The agents evidently act in large part by providing young epidermal cells in quantity to the virus, as also by inducing a richer vascularization than ordinary in support of the papillomatous proliferation It is possible that they also act by providing especially susceptible cells The implications of the findings are discussed

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EXPLANATION OF PLATES

All of the specimens were stained with eosin and methylene blue

The photographs were made by Mr Joseph B Haulenbeek

Plate 2

Fig 1 Normal skin, from the abdomen of a normal domestic rabbit It has been overstained with hematoxylin to make the thin epidermal layer more evident × 18

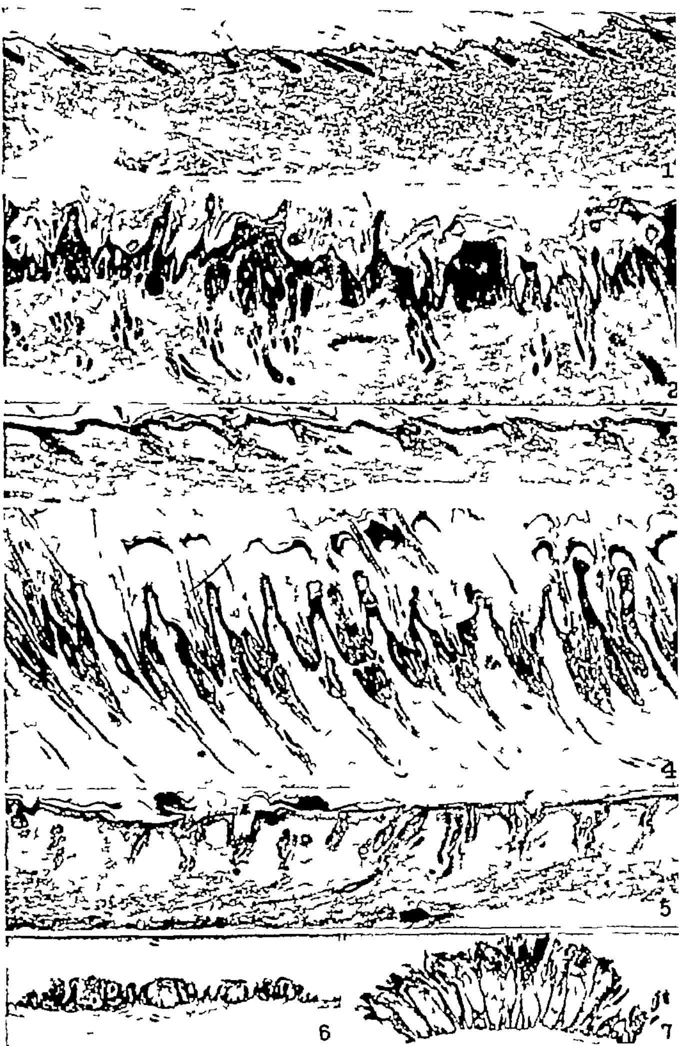
Fig 2 Portion of an area of abdominal skin which had been tarred twice a week for 2 weeks Biopsy 2 days after last application × 18

Fig 3 Skin treated with turpentine and acetone The mixture had been applied five times at 2 day intervals Biopsy 24 hours after last treatment × 18

Fig 4 To show the skin alterations induced by four applications at 2 day intervals of a saturated solution of Scharlach R in benzene containing 0 3 per cent methylcholanthrene × 18

Fig 5 Illustrating the marked hyperplasia of normal skin after scarification Specimen taken 8 days after scarification × 18

Figs 6 and 7 Cross section of the papillomatous masses formed after broadcast tattoo inoculation of normal skin (Fig 6) and of methylcholanthrene treated skin (Fig 7) of the same rabbit The growths were removed and sectioned 42 days after virus inoculation Only a portion of the mass on the methylcholanthrene treated skin is shown × 2½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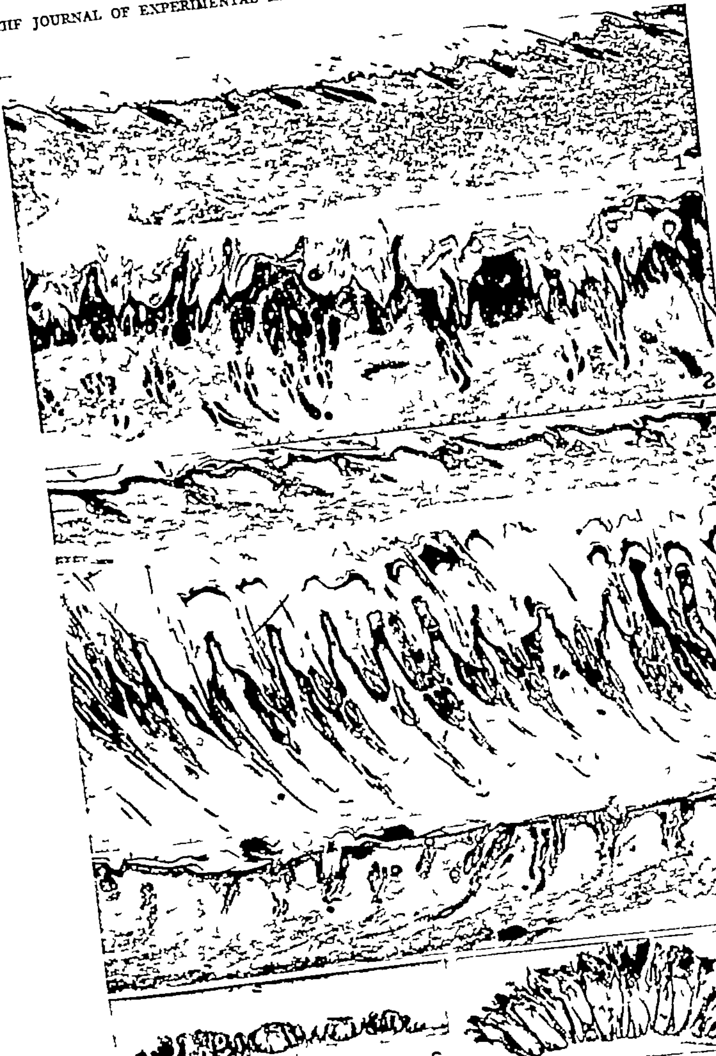
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Plate 3

Figs 8 to 12 To illustrate the changes induced by scarification of methylcholanthrene treated skin All the specimens were taken from the same rabbit

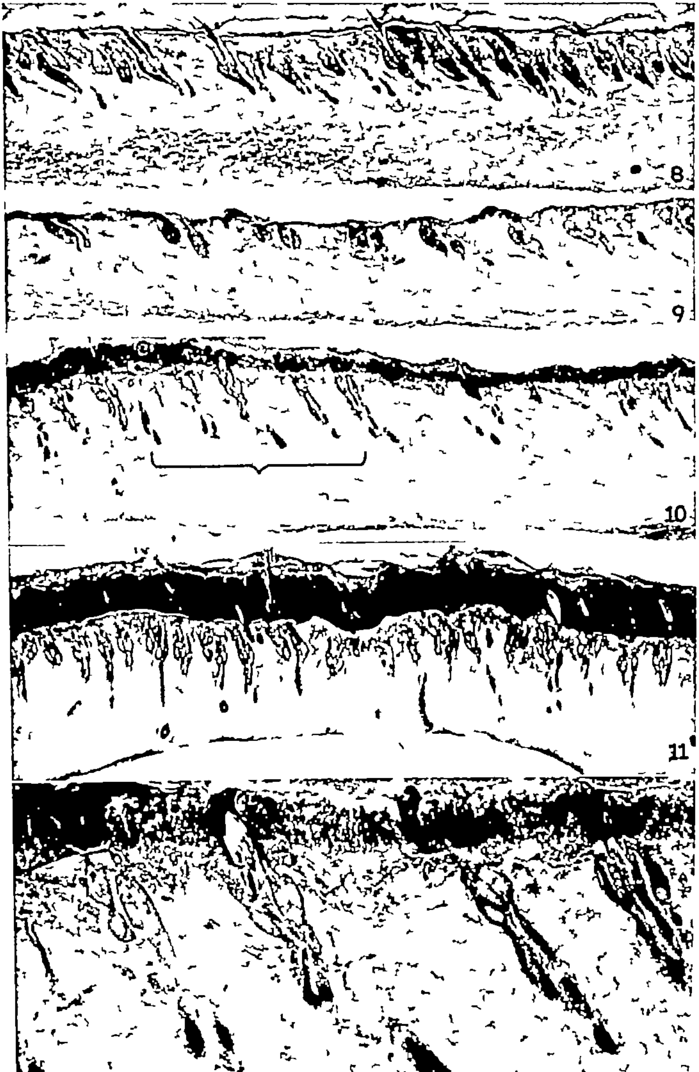
Fig 8 Methylcholanthrene treated skin before scarification 0 3 per cent methylcholanthrene in benzene had been applied to it four times at 2 day intervals Biopsy 24 hours after last treatment × 18

Fig 9 5 hours after scarification The surface epithelium has been almost completely removed together with some connective tissue and the upper portion of the hair follicle shafts A scab is forming on the surface × 18

Fig 10 24 hours after scarification The scab is unusually thick as result of superficial necrosis involving both the connective tissue and hair follicle shafts From the latter epithelial regeneration is already taking place, the new cells extending between scab and connective tissue The bracketed region is shown at greater magnification in Fig 12 × 18

Fig 11 48 hours after scarification Epithelial regeneration is now complete and the denuded surface is covered with markedly hyperplastic epidermis The sebaceous glands are also hyperplastic and many are distended with secretion Hence their prominence as compared with those of Fig 8 × 18

Fig 12 The bracketed region of Fig 10 at higher magnification The epithelium is spreading laterally from the shafts of the hair follicles to form umbrella like structures Some of the new epidermal cells can be seen extending irregularly into crevices in the fibrous corium × 60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RED CELL AND PLASMA VOLUMES (CIRCULATING AND TOTAL) AS DETERMINED BY RADIO IRON* AND BY DYE‡

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The total blood volume is usually calculated from the plasma volume and the venous hematocrit with the use of the formula

$$\text{Total blood volume} = \frac{\text{Plasma volume}}{(1 - \text{venous hematocrit})}$$

The plasma volume has commonly been determined by one of the several dye dilution methods and as performed has been assumed to be accurate to within 5 per cent. The venous hematocrit has been assumed to be representative of the average hematocrit of all the blood in the vascular system Application of the principles of hydrodynamics to the problems of blood flow and blood volume indicates that neither of these assumptions is valid The experimental data to be presented confirm the fact that the blood volume values calculated from the plasma volume and venous hematocrit are subject to considerable systematic error

In the observations subsequently to be described, we shall demonstrate by several techniques that the mass of red blood cells in the body is approximately 75 per cent of the commonly accepted amount, that few of these cells are immobilized in "reservoirs," that the mixing time for erythrocytes is much more rapid than that for dyes, and that the usually accepted times for mixing of dyes in the vascular system probably represent mixing in the "axial stream" only, and not in the entire contents of the vessels

About 100 years ago Peclet (11, 15) pointed out that when a liquid moves over a solid surface a sluggishly moving fluid film exists on the surface of a stationary fluid film. The character of both films is entirely different from that of the main body of moving fluid and they form a zone between the rapidly moving fluid and the wall,

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* We are indebted to the members of the Radiation Laboratory at Berkeley California, and in particular to Dr E. O Lawrence and Dr M D Kamen for the radioactive iron used.

‡ We are indebted to Eli Lilly and Company for aid in conducting this work.

acting toward the transportation of heat, vapor, and matter as though they were a separate material. The presence of these films has been verified by many investigators (10), and their properties are controlling factors in many processes. Their characteristics receive important engineering consideration, and they deserve equally as much attention from the physiologist.

An instance of the importance of these films is encountered in a consideration of the mechanics of mixing of particulate matter in a vessel. A plot of the velocity of a fluid against its distance from the wall of the containing vessel shows that there is no motion of the fluid at the boundary wall (13), and that with progression toward the center of the tube the velocity of the motion increases. Near the wall of the vessel the fluid flow is in a path parallel to the wall, and there is no component of velocity at right angles to the latter. Approaching the center of the vessel, however, the fluid elements begin to travel in forward moving spirals although the "net flow" is still parallel to the wall. The path of any given particle now has a large component of velocity at right angles to the vessel wall. Thus, from a condition of "viscous," or "straight line" flow near the wall we have progressed to "turbulent" flow in the center. These two types of flow exert a profound and very different effect upon the mixing of the neighboring fluid layers.

Applying these principles to the problem of hemodynamics it is evident that when blood flows through a blood vessel, two films of plasma exist along the wall of the vessel, one without motion, and the other with a sluggish motion parallel to the wall of the blood vessel. The flow of this film is "viscous" or "straight line," and there is no turbulence or mixing between these films and the rest of the contents of the vessel. Microscopic inspection of small blood vessels shows that these films are for the most part devoid of erythrocytes.

The thickness of the surface films varies inversely with the velocity of flow of the main body of the fluid contents, and the percentage that they constitute of the total fluid contents of a vessel varies inversely with the diameter. In the blood vascular system as the arteries and arterioles become smaller and approach the capillary bed there is no increased velocity of blood flow, but an actual *decrease* in *velocity*. There is also a great increase in the number of vessels and a decrease in their diameter. Therefore the surface films might be expected to constitute a progressively increasing proportion of the total contents of small arteries, arterioles, and capillaries. That this should be the case is demonstrated by the following formula.

The total volume of the fluid in a unit length of vessel is πr_1^2 where r_1 is the radius of the vessel. If t is the thickness of the *combined stationary and sluggish films*, which consist of plasma only, then the radius of the body of fluid in axial flow is $r_1 - t = r_2$, and the volume of fluid in the axial stream is πr_2^2. The volume of plasma in the films is then $\pi r_1^2 - \pi r_2^2$. The fraction (f) of total plasma in the films is then

$\dfrac{\pi r_1^2 - \pi r_2^2}{\pi r_1^2}$, eliminating π

(1) This may be expressed as $\dfrac{r_1^2 - r_2^2}{r_1^2} = f$

(2) Equation (1) may be expanded to $\dfrac{(r_1 - r_2)(r_1 + r_2)}{r_1^2} = f$

(3) Now since $r_1 - r_2 = t$ which is constant for a constant velocity of fluid flow we can substitute (3) in (2) $\frac{(r_1 + r_2)k}{r_1^2} = f$ and, since r_1 approximates r_2 where t is small, we have $\frac{2r_1\ k}{r_1^2} = f$ or $\frac{r_1 \times k_1}{r_1^2} = f$ eliminating one r_1 $\frac{k_1}{r_1} = f$ Thus, since f is shown to be indirectly proportional to the radius, as the vessel becomes smaller the fraction of plasma in sluggish movement increases. It is obvious that this relationship does not hold in capillaries where the diameter approaches that of a single red cell However, Zweifach and Kossmann (18) found that the individual capillaries vary considerably in size, the fluctuations noticed being from 3 to 10 micra in diameter

It has been observed by many investigators that rapid plasma flow may occur in vessels apparently too small to permit passage of red cells

The influence of these surface films on determinations of blood volume was first pointed out by Hooper Smith Belt, and Whipple (9), who recognized that the hematocrit of capillary blood was considerably lower than that of venous blood due to the existence in small vessels of a central axial stream ' of cell rich blood and a peripheral, relatively cell free still space Using the carbon monoxide inhalation and Welcker viviperfusion methods to determine the volume of erythrocytes ('direct methods ') they demonstrated that the total mass of erythrocytes was 20 to 30 per cent less than the values obtained by calculations based on the plasma volume and venous hematocrit.

Fahraeus (2) emphasized the fact that the blood flowing through a capillary has a much lower ratio between cells and plasma than the blood which flows from its cut end an observation which is explained by the relatively great velocity of flow of the cell rich axial stream and the slow flow of the peripheral films.

Using erythrocytes tagged ' with radioactive iron, and determining their dilution in the circulating blood Hahn Balfour Ross Bale, and Whipple (6) confirmed the fact that the total mass of red blood cells in the body is approximately 25 per cent less than that indicated by the plasma volume-venous hematocrit calculations.

Stead and Ebert (14) recently arrived at the same conclusion by demonstrating the discrepancy between hematocrit and hemoglobin changes produced by bleeding and the amounts of blood removed.

The surface film-axial stream relationship existing in the blood vessels and the differences in type of particle motion within them not only influence the distribution of erythrocytes in these vessels but also operate to retard the passage of dyes from the rapidly moving turbulent axial stream into the sluggishly moving and stationary peripheral films. This difference in type of mixing has been neglected in the usual plasma volume determination and has introduced considerable error into the procedure, an error which in turn has been reflected in the blood volume estimation.

Methods

Unexercised, healthy normal or anemic dogs were used for all experiments. To avoid lipemia the food was withheld for 18 hours before plasma volume determinations were made. Plasma volumes were determined by a modification of the brilliant vital red procedure (9) The amount of dye injected was sufficient to produce such a

concentration in the plasma sample that the colorimeter reading was within 10 per cent of the standard diluted dye reading. A single sample of plasma was secured 4 minutes after administration of the dye, care being exercised to avoid stasis in the vein and hemolysis. The anticoagulant used was 1.4 per cent sodium oxalate solution.

The mass of red blood cells was determined by four techniques: (a) by vivi-perfusion and determination of the total amount of hemoglobin removed (9), (b) by determination of the amount of tagged blood cells which had to be withdrawn to remove completely from the circulation all of the radioactive iron tagged cells (which had previously been built up in the circulation by methods described (7) elsewhere), (c) by determining the dilution of radioactive tagged cells after removal of a known quantity of blood (and radioactive tagged cells) when regeneration of blood had restored the original hematocrit, (d) by determining the dilution in the circulation of injected radioactive tagged erythrocytes (6).

The technique of the modified Welcker viviperfusion method has been described elsewhere (16). The perfusate was collected in isotonic sodium oxalate solution, and the hematocrit of an aliquot determined. Total iron content or radioactive iron concentration and hemoglobin content were determined on packed red cells, (since the use of whole blood for the latter procedure may lead to erroneous results (17)). Hemoglobin determinations were performed according to the method of Newcomer (12).

Determination of the red cell volume by either of the depletion techniques (method 2 or 3) was accomplished as follows. An iron depleted anemic dog was fed a single dose of radioactive iron and regeneration of hemoglobin was allowed to proceed until the concentration of this material in the blood was nearly constant. The animal was then bled repeatedly over a period of several weeks until all of the radioactive tagged cells were removed from the circulation. The radioactivity of the blood removed at each bleeding was determined, and the total activity of all the removed blood calculated. The total red blood cell volume was then calculated by dividing the total activity removed by the initial concentration of radioactivity per unit volume of cells.

A modification of this procedure consisted in bleeding the animal several times and allowing blood regeneration to restore the hematocrit to the same level that existed prior to the hemorrhages. Assuming the red blood cell volume to be the same at the same hematocrit level, this volume was then calculated by dividing the total radioactivity removed in the bleedings by the difference in concentration of the isotope in the circulating blood before bleeding and after regeneration.

The donor cell dilution method of determining red blood cell volume has been described briefly elsewhere (6). It has distinct advantages over other methods of cell volume determinations. It can be performed on normal animals or animals with any degree of anemia. The conditions under which it may be carried out are physiological and do not disturb the normal hemodynamics or vascular system. The tagged cells are not subject to loss from the vascular system (as are dyes and carbon monoxide). Erythrocytes "tagged" or "labelled" with radioactive iron were drawn from a donor dog in whom hemoglobin regeneration with radioactive iron had been produced (7). These cells, containing a known amount of radioactive iron, were then administered intravenously (in a period of 30 seconds) to the dog under study (whose own cells

contained none or a known concentration of radioactive material) The degree of dilution of the radioactive tagged cells was determined on blood samples removed at 2, 4, 6, 10, and 15 minutes and at daily intervals following the injection Coagulation of these samples was prevented with isotonic sodium oxalate, they were centrifuged 35 minutes at 2500 R.P.M., the cell-plasma ratio was determined, and the cells were subjected to wet ashing and radioactivity determination as described elsewhere (4, 5) The red blood cell volume was then calculated by dividing the total radioactivity in the injected donor erythrocytes by the radioactivity per unit volume of cells in the sample.

TABLE I

Red Cell Volume and Average Body Hematocrit by Viviperfusion Method

Dog	Weight	Plasma volume by dye	Cell volume calculated by dye	Cell volume determined	Blood volume determined	Jugular hematocrit	Average hematocrit	Ratio cell volume determined to cell volume by dye	Ratio average hematocrit to jugular hematocrit
	kg	*ml.*	*ml.*	*ml*	*ml.*	*per cent*	*per cent*	*per cent*	*per cent*
33-329	15.6	700	660	400	1100	49.0	36	61	74
40-167	14.5	680	725	495	1175	51.3	42	68	82
39-161	8.3	320	310	285	605	48.6	47	92	97
38-320	14.9	760	240	160	920	23.5	17	67	72
37 186	6.4	285	155	130	415	37.4	32	84	85
Pup 3-A		235	145	115	350	37.5	32	79	85
" 4-A		285	170	115	400	36.9	29	68	79
" 5-A	6.2	295	180	125	420	38.0	30	70	79
" 6-A	6.4	375	190	115	490	33.4	24	61	72
" 1 B*	2.1	165	67	30	195	28.6	15	45	52
" 1-C	10.3	395	280	210	605	41.2	35	75	85
" 2 C	4.7	285	145	120	405	32.6	30	83	92
" 5-E	16.0	720	725	570	1290	50.0	44	72	88
Average								71	80

* 1 B emaciated.

The radioactive isotope of iron, Fe^{59} used for these experiments was prepared by deuteron bombardment of iron and had a half period of 47 days. Preparation of samples for radioactivity determination has been described (4, 5)

EXPERIMENTAL OBSERVATIONS

1 The Erythrocyte Volume As Indicated by Viviperfusion

When properly performed viviperfusion very effectively removes practically all red cells from the body The hematocrit of the perfusate is usually below 1 per cent terminally, and the tissues (with the exceptions of the spleen and bone marrow) are bloodless at autopsy and microscopically As shown by the iron analyses of Hahn and Whipple (8), the total mass of erythrocytes left in the body after viviperfusion does not amount to more than 1 to 2 per

cent of the total cell mass Thus the values for erythrocyte volumes shown in Table I are subject to an error of not more than 5 per cent Comparison of the values for red blood cell volume as determined by viviperfusion is made with the values calculated from the plasma volume-jugular hematocrit It is evident that in every instance the latter method gives results 15 to 30 per cent higher than those obtained by vivi perfusion

2 The Erythrocyte Volume As Determined by Depletion of Radioactive Iron Containing Cells

In Table II the volume of erythrocytes as calculated from the plasma volume and jugular hematocrit is compared with the volume as determined by depletion of radioactive iron containing cells over a period of weeks by repeated

TABLE II

Comparison of Total Red Blood Cell Volumes As Calculated from Plasma Volumes and Determined Directly by Radio Iron Hemoglobin

Dog	Red cell volume determined directly	Red cell volume calculated from plasma volume	Plasma volume by dye	Jugular hematocrit	Average body hematocrit	Ratio red cell volumes
	ml	*ml*	*ml*	*per cent*	*per cent*	*per cent*
By depletion of radio iron in circulation by repeated hemorrhage						
39 196	350	460	575	43 2	37 8	0 76
38-170	300	490	475	50 5	38 8	0 61
By change in concentration of radio iron in circulation						
38-137	270	405	590	40 7	31 4	0 67
39 133	215	310	455	39 7	32 0	0 69
37-202	160	280	570	31 6	22 0	0 57
37 202	120	200	810	18 6	12 9	0 60

hemorrhages Since the animals used in this type of experiment were iron deficient and only a single feeding of a small amount of iron was given, all of the radioactive isotope was present in circulating erythrocytes at the beginning of the experiments Complete removal of these tagged cells reasonably can be assumed to indicate complete removal of all the cells present in the vascular system at the beginning of the experiment The mass of cells as determined by this procedure is found to be 24 and 39 per cent lower than the values calculated from the plasma volume and jugular hematocrit

3 The Erythrocyte Volume As Indicated by the Regeneration Dilution of Radioactive Iron Containing Cells

The volume of erythrocytes determined by regeneration dilution of radioactive iron containing cells following removal by bleeding of a known quantity of blood is compared with the values calculated from plasma volume and jugular

hematocrit in Table II The latter method gives values considerably higher than those obtained with the dilution method

4 *Erythrocyte Volume Determined by Injection of Tagged Cells*

Table III shows the erythrocyte volume as indicated by determinations of dilution of injected radioactive iron containing cells The rapidity with which these cells are mixed with those of the entire vascular system is evidenced by the almost identical values obtained after 2 minute and 24 hour mixing periods It is improbable that such rapid and complete mixing would

TABLE III

Circulating and Total Red Cell Mass Determined by the Radioactive Donor Cell Method

Dog	Activity injected cells	Activity in recipient cells						Average activity concentration 4, 6, and 10 min.	Circulating red cell volume	Total RBC volume
		Counts/min* per 100 ml RBC								
		2 min.	4 min	6 min.	10 min.	24 hrs.	Other			
	counts/min							*counts/min./RBC %*	*ml*	*ml*
39-307	905	233	196	209	206	222	208 (8 days)	204	445	405
40-149	705	231	183	187			208 (5 days)	185	380	340
36-196	1260	438	427	430	440		453 (3 days)	432	292	278
39-144	128	58	48	51	42	54	48 (4 days)	47	272	237
39-88	761	162	168	157	168	148	148 (3 days)	164	465	513
39-194	1135	310	286	289	302			292	389	
39-193	491	98	87	88	78			84		
39 299	558		334	386		311		360	155	180
40-183	865				187	198			460	440
39-196	1680				398	410			420	410
4-E	1680				537	480			315	350
Average									355	350

* Scale of four Geiger counter

occur if any considerable proportion of the total volume of erythrocytes were immobilized in splenic sinusoids or elsewhere in the body

DISCUSSION

Our results indicate that the total erythrocyte volume as determined by direct procedures is 10 to 40 per cent less than the volumes derived by the plasma jugular hematocrit method The total blood volume can be ascertained by adding the cell volume (determined by radioactive tagged erythrocyte method) and the plasma volume (determined by dye dilution method) The average hematocrit value for the entire contents of the vascular system can then be determined by dividing the directly determined cell volume by the

total blood volume This average hematocrit is invariably considerably (about 20 per cent) lower than that of the large vessels To compensate for the relatively greater number of cells present in the large vessels, the cell-plasma ratio in the small vessels must, therefore, be even less than that of the average The theoretical explanation for this finding has been presented above in the discussion of the peripheral plasma films

No significant fraction of the erythrocyte volume can be immobilized in the spleen, liver, or other regions, since the volume of cells in rapid circulation (as indicated by the values for erythrocyte volume determined after only 2 or 4 minutes mixing of injected, radioactive tagged cells) is practically identical with the total volume of erythrocytes (indicated by the volume found after 24 hour, or longer, periods of mixing) It is improbable that such complete mixing could occur so rapidly if many cells were immobilized, and it appears that practically all red blood cells in the dog are in active circulation Similar conclusions have recently been drawn by Ebert and Stead (1)

Since there is no evidence that any considerable fraction of the erythrocyte volume is immobilized, it may be concluded that all the red blood cells of the dog are in active circulation and that the *circulating erythrocyte volume* is practically identical with the *total erythrocyte volume* Since the cell-plasma ratio of the rapidly flowing axial streams in various vessels is fairly constant, the jugular hematocrit may be assumed to be representative of this ratio for the rapidly circulating ("axial stream") blood, and the actual *rapidly circulating blood volume* may be calculated by dividing the cell volume (as determined with the donor cell method) by the jugular hematocrit The rapidly circulating blood volume as determined in this manner is considerably lower than the total blood volume ascertained by the addition of the cell volume (directly determined by the isotope dilution method) and the plasma volume (from the dye procedure) Tables IV and V summarize these differences

The amount of plasma in rapid circulation ("rapidly circulating plasma volume") can be calculated from these values by subtracting the determined cell volume from the calculated rapidly circulating blood volume In similar fashion, if the value for plasma volume as determined by the dye method is accepted as representative of the *total* plasma volume, the difference between this value and "the rapidly circulating plasma volume" is a measure of the fraction of the plasma in relatively sluggish circulation This fraction amounts to 6 to 37 per cent in the series of animals listed in Tables IV and V

The previously described laws of hydrodynamics apply to the passage of dyes between the various fluid layers of the circulating blood as well as to the mixing of erythrocytes The passage of dyes from the central turbulent stream into the peripheral films occurs only by the slow process of diffusion Since we have demonstrated that these peripheral films may constitute a very considerable proportion (21 per cent) of the total amount of plasma, appreciable

TABLE IV

The Partition of Plasma in the Vascular System by Radio Iron

Dog	Weight	Jugular hemato-crit	RBC volume	Blood volume circulating	Plasma volume circulating	Plasma volume by dye	Plasma volume difference	Total plasma volume in slow circulation
	kg	*per cent*	*ml.*	*ml*	*ml.*	*ml*		*per cent*
39-307		51 4	445	865	420	480	60	13
39-144	9 5	44 0	255	580	325	485	160	33
39-88		53 7	465	865	400	520	120	23
39-194	17 0	40 0	389	970	580	720	140	19
40-149	10 0	42 4	380	900	520	525	5	1
39-299	14 3	21 3	155	730	575	645	70	11
40-183	13 5	48 0	460	960	500	585	85	15
39-196	15 1	38 2	420	1100	680	645	−35	
4-E	16 0	39 4	315	800	485	725	240	33
33-329	15 6	49 0	400	815	415	700	285	41
3-A		37 5	115	305	190	235	45	19
4-A		36 9	115	310	195	285	90	32
5-A	6 2	38 0	125	330	205	295	90	31
37 186	6 4	37 4	130	350	220	285	65	23
38-320	14 9	23 5	160	680	520	760	240	32
40-167	14 5	51 3	495	1160	665	680	15	2
39-161	8 3	48 6	285	585	300	320	20	6
36-196		39 0	290	745	455	565	110	19

TABLE V

Partition of Plasma in the Vascular System at Various Hematocrit Levels

Dog	Weight	Hemato-crit	RBC volume deter mined	Blood volume circulating	Plasma volume circulating	Plasma volume by dye	Plasma volume difference	Total plasma volume in slow circulation
	kg	*per cent*	*ml.*	*ml.*	*ml*	*ml*	*ml.*	*per cent*
40-183	13 5	53 2	525	985	460	580	120	20
		48 0	460	960	500	585	85	15
		29 9	212	710	498	745	247	33
		27 8	202	725	513	700	175	25
39-196	15 1	38 2	420	1100	680	645	−35	
		36 5	345	945	600	640	40	6
		27 0	215	795	580	820	240	29
		26 9	235	875	640	820	180	22
		23 8	200	840	640	700	60	9
4-E	16 0	39 4	315	800	485	725	240	33
		27 6	230	830	600	955	355	37
		27 4	270	985	715	955	240	25
		22 6	205	905	700	815	115	14
39-320	10 0	49 2	260	530	270	370	100	27
		25 2	155	615	460	490	30	6
38-137	10 7	48 7	410	840	430	480	50	10

difference in time of mixing in the axial stream and total fluid may be predicted. Also, since dyes are presumably lost from the circulation by various processes, if the time interval between samples is great enough to allow for complete diffusion, results obtained by these methods are likely to be falsely high. Theoretically, there should be a complex type of time curve for the concentration of dye in the blood, since at least three processes are taking place simultaneously.

1. Turbulent mixing of the dye in the large vessels and in the axial stream of small vessels.

2. Diffusion of the dye into the peripheral sluggishly moving and stationary plasma films.

3. Loss of dye through removal by the reticulo-endothelial system and excretion through the biliary tract.

Conceivably each of these processes might be demonstrated graphically if concentration of dye is plotted against time, providing that all or any two did not have rates of such a character that the curves approached similarity despite varying order of functions. Actually such differences in the curves of plasma volume determinations can be demonstrated. Inspection of the curves of various investigators using the Evans blue (T-1824) as well as the brilliant vital red dye, indicates at least two phases of distribution of dye in the vascular system, and personal observations indicate that a third may well be present. Gibson and Evans (3) divide their curve into two components which they term the "mixing phase" and the "disappearance phase." The intersection of the tangents of these curves is assumed to represent the true mixing time of the dye in all the plasma, and the point of intersection of the extrapolated tangent with the ordinate is claimed to indicate the value for the optical density (a function of the true plasma volume) at the time of dye injection. More careful study of the character of these curves indicates that three distinct phases may be demonstrated. Not only the slopes, but also the shapes of these curves differ, the first two (comprising the mixing curve) being exponential, while the disappearance curve is almost linear.

The two phases of the mixing curve are produced by the mixing in the axial stream, and the diffusion mixing of the peripheral plasma films. Careful analysis indicates that the latter process may frequently extend over a time much longer than that commonly assumed to allow complete mixing. Calculations based on dye dilutions at periods before complete mixing has occurred yield plasma volumes which are low. In spite of this fact, however, the plasma volumes so determined are still considerably higher than the rapidly circulating plasma volumes.

The rapidly circulating and sluggishly flowing plasma fractions are not to be considered as separate entities, inasmuch as there is constant (although slow) interchange between them. They do, however, greatly influence consideration of blood flow and blood volume.

SUMMARY

1 Application of the principles of hydrodynamics to the problem of blood flow and blood volume indicates that the calculation of blood volume and cell volume from the venous hematocrit and plasma volume (as determined by the dye method) is subject to considerable error

2 This conclusion is borne out by determinations of total cell volume by viviperfusion and with the use of radioactive iron tagged erythrocytes, which have shown the erythrocyte volume to be only 70 to 75 per cent of the volume indicated by the previously mentioned calculations

3 The average hematocrit of the entire vascular system is considerably lower than the hematocrit of the large vessels, and the cell plasma ratio of the smaller vessels is still less.

4 In the dog there are no considerable stores of immobilized erythrocytes, and the total erythrocyte volume and circulating erythrocyte volume are identical

5 The "rapidly circulating blood volume" can be determined by dividing the erythrocyte volume by the venous hematocrit, and is found to be considerably less than the total blood volume.

6 The concept of the "rapidly circulating plasma volume" is introduced, and it is found to be approximately 80 per cent of the total plasma volume

7 The volume of plasma in the peripheral, cell free, sluggishly moving plasma films plus that contained in small vessels in which no red cells are present, is also determined and found to be approximately 20 per cent of the entire plasma volume

8 The existence and magnitude of these fractions of the blood plasma volume should receive consideration in studies of blood flow and blood volume.

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INFLUENCE OF AGE ON SUSCEPTIBILITY OF MICE TO ST LOUIS ENCEPHALITIS VIRUS AND ON THE DISTRIBUTION OF LESIONS*

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PLATE 4

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Evidence has accumulated that young mice are more susceptible than older ones to many neurotropic viruses if they are inoculated by a peripheral route The most significant decline in susceptibility apparently occurs as mice pass 2 weeks of age, but there is uncertainty concerning the factors which contribute to this age difference Andervont (1) showed that 2 week-old mice were less resistant than adults to intracutaneous inoculation of herpetic virus into the abdominal skin and Theiler (2) demonstrated the greater susceptibility of young mice to the intraperitoneal inoculation of the virus of yellow fever

Several explanations have been advanced to account for the greater susceptibility of young mice to peripherally inoculated virus Sabin and Olitsky (3, 4) suggested that the myoneural junction or specialized nerve endings might impede the progress of the virus in adult mice inoculated intramuscularly with the virus of vesicular stomatitis To explain the difference in susceptibility when the same virus was introduced by the olfactory route they offered the explanation that a central barrier preventing the passage of the virus existed in adult mice between the primary olfactory centers of the telencephalon and the remainder of the brain King (5), studying Eastern equine encephalomyelitis, believed it probable that the increased resistance of older mice to peripheral inoculation could be explained by a change in the brain tissue itself such as might be brought about by increased cell maturity,—myelination, change in water content, and in reaction to specific and non-specific injury Following intramuscular inoculation of the virus of Western equine encephalomyelitis Sabin and Olitsky (6 7) observed in mice 2 weeks of age, a high incidence of symptoms of encephalitis with lesions indicative of invasion along the olfactory or other pathways without evidence of either diffuse hemato-encephalitic spread or progression by way of the local nerves On the other hand a high incidence of symptoms attributable to ascending infection was observed in mice 3 weeks of age. At one month of age and beyond, many mice were entirely resistant following intramuscular inoculation

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They attributed the difference between the first two groups to the greater permeability of certain blood vessels of the young animal for the virus, and the difference between the latter two groups to changes in the muscle or specialized nerve endings

A difference in invasive capacity between fresh and fixed viruses, emphasized by the studies of King (5) and Casals (8), may also play a rôle in determining the greater susceptibility of young animals Other factors of possible significance in this respect are the ability of a virus to multiply in the blood (Hurst (9), equine encephalomyelitis, monkey, and guinea pig) or other tissues of the body, and the rate at which antibodies are produced (Morgan (10), Eastern equine encephalomyelitis, mice) at different ages

The influence of age on the susceptibility of mice inoculated intraperitoneally with the virus of St Louis encephalitis has been determined in the present investigation The explanation for the difference in susceptibility between young and old animals has been sought in a study of the distribution of lesions in mice between 2 and 8 weeks of age killed at intervals following intraperitoneal inoculation The distribution of the lesions has been compared with that which followed intranasal inoculation in adult mice Special attention has been directed to two possibilities (1) that evidence of a direct hematogenous invasion of the C N S might be found in young mice, (2) that young mice inoculated intraperitoneally, in contrast to older mice, might show a distribution of lesions coinciding with that found when the olfactory route of inoculation was used

Material and Methods

The Hubbard Strain of St Louis encephalitis virus, isolated in 1937, was used in this study At present the virus has a constant infective titer as great as 10^{-7} when measured by intracerebral inoculation of mice 2 months of age On some occasions the infective titer so measured reaches 10^{-8} When these experiments were begun, the virus had been carried through approximately 100 successive passages in the mouse brain The material for injection was prepared in a uniform manner for all experiments Brains removed from mice in the convulsive stages of encephalitis were kept in the freezing unit of the refrigerator (−15° to −20°C) for 1 to 2 days Brains, shown to be bacteriologically sterile, were ground without abrasive and suspended in sufficient nutrient broth (pH 7 4) to make a 10 per cent suspension Following centrifugation at 200 R P M for 2 minutes the supernatant fluid was drawn off and used to make further tenfold dilutions in nutrient broth For a given route of inoculation the same quantity of fluid inoculum was used irrespective of the size of the mouse

For the susceptibility studies, groups of mice of different ages were usually inoculated in the same experiment When this was not possible because of the difficulty of having on hand a sufficient number of mice of all the required ages, the titer of the virus was confirmed by the intracerebral inoculation of mice approximately 2 months of age Swiss mice obtained from a single source were used with the following exception Part of the mice in the 2 weeks age group were white mice of the "Old Buffalo strain However, the latter strain and the Swiss mice which we have used

have shown the same degree of susceptibility to the virus of St. Louis encephalitis when inoculated intracerebrally

For the anatomical studies the virus was instilled intranasally and inoculated intraperitoneally Usually the mice of a single experiment were killed at spaced intervals preceding the time at which it might be expected that clinical signs of the disease would develop Several mice of each group were kept until symptoms had appeared. The brains were fixed in a mixture of aqueous corrosive sublimate and absolute alcohol, embedded in paraffin, and sectioned serially in a frontal plane at 20 μ Every third slide of each series was stained with cresyl violet, the maximum distance separating stained slides being 0 4 mm With few exceptions representative levels of the spinal cords were cut in all mice of the series inoculated intraperitoneally Other cords were preserved and cut when it appeared desirable.

164 brains were obtained, 25 from mice 6 to 8 weeks of age instilled intranasally with 10^{-3} or 10^{-4} dilution of virus and 139 from mice 2 to 8 weeks of age inoculated intraperitoneally with 10^{-1} and higher dilutions of virus

Susceptibility to Intracerebral, Intranasal, and Intraperitoneal Inoculation of Virus

Intracerebral Inoculation—In each of two experiments groups of 4 mice, 3 weeks, 6 weeks, and 15 months of age were inoculated intracerebrally with 0 03 cc of tenfold dilutions of virus in broth In a 3rd experiment 2 groups of mice 8 weeks and 10 months of age were inoculated with corresponding dilutions of virus Finally two groups of mice, 2 weeks and 3 months of age, were inoculated in a similar manner As shown in Table I, mice of all ages were uniformly infected when they received 10^{-7} dilution of virus intracerebrally When the titer of the virus reached 10^{-8} approximately the same number of the mice died in all groups

Intranasal Inoculation—Groups of 8 mice, 3 weeks, 6 weeks, and 15 weeks of age were inoculated intranasally with 0 03 cc of dilutions of virus in broth. Again no difference in the response of the various age groups could be distinguished (Table II) At all ages at least a part of the mice died following intranasal instillation of the 10^{-4} dilution of the virus while only an occasional mouse in any age group showed evidence of infection following instillation of the 10^{-8} dilution The same results were obtained in other experiments using these three age groups, and also in one experiment in which a group of mice 10 months of age was used

Intraperitoneal Inoculation—In contrast to the intracerebral and intranasal routes, the age of the mice influenced the results when the virus was inoculated intraperitoneally, (Table III) Tenfold dilutions of the virus in broth were injected in 0.25 cc amounts by this route When groups of mice 3, 6, and 15 weeks of age were used, most of the mice in the oldest age group remained well following the inoculation of a 10^{-1} dilution of the virus Of mice 6 weeks of age, a slightly greater number succumbed to this amount of virus but only an

occasional mouse died following the inoculation of the 10^{-2} dilution On the other hand all mice 3 weeks of age succumbed when inoculated intraperitoneally

TABLE I

Susceptibility of Mice of Different Ages to the Virus of St Louis Encephalitis Inoculated Intracerebrally

Age of mice	Dilutions of virus		
	10^{-4}	10^{-7}	10^{-8}
3 wks	(1)* 5‡ 5 5	4 4 5 5	4 4 5 5
6 wks	(1) 4 5 6	4 4 4 5	4 5 5 5
15 wks	4 4 4 5	4 4 5 5	4 4 5 5
3 wks.	4 4 4 5	4 5 5 5	5 S§ S S
6 wks	4 5 5 6	4 5 5 6	5 S S S
15 wks	(1) (1) 4 5	5 5 5 6	5 5 S S
8 wks		5 5 6	7 S S
10 mos		5 6 6 6 6 9	9 12 18 S S S
14 days	———	4 4 5 5	4 4 6 S
8 wks.	4 4 5 5	5 5 5 5	5 6 S S

* ( ) = death from unknown cause

‡ Numbers refer to day of death following inoculation

§ S = survival of mouse.

TABLE II

Susceptibility of Mice of Different Ages to the Virus of St Louis Encephalitis Inoculated Intranasally

Age of mice	Dilutions of virus		
	10^{-2}	10^{-4}	10^{-6}
3 wks	5 6 7 S	5 6 6 6	S S S S
6 wks	5 5 6 7	6 7 S	S S S S
15 wks.	5 6 6 7	6 6 6 7	7 S S S
3 wks.	6 6 7 7 7 7 9 S	(2) 6 7 8 8 10 S S	———
6 wks	5 6 6 6 7 9 10 10	7 S S S S S	———
15 wks	5 6 7 8 10 S S S	7 7 7 9 9 9 S S	———
3 wks	6 6 7 7	6 S S S	S S S S
6 wks.	6 6 6 7	6 6 7 S	8 S S S
15 wks	6 6 7 S	6 7 7 S	7 S S S
10 mos.	6 6 7 7 7 7	7 7 8 8 S S	7 S S S S S

with dilutions of 10^{-1} and 10^{-2} of the virus, and an occasional mouse receiving either the 10^{-3} or 10^{-4} dilution died In comparison with the mice 3 weeks

of age, those 2 weeks of age were much more susceptible to the virus when it was inoculated intraperitoneally, succumbing to dilutions of the virus as great as 10^{-6}

In summary these results indicate that no significant age difference in susceptibility is apparent in mice inoculated intracerebrally or intranasally, whereas mice inoculated intraperitoneally show increasing resistance with age, the most striking difference appearing between the 2nd and the 3rd week

TABLE III

Susceptibility of Mice of Different Ages to the Intraperitoneal Inoculation of the Virus of St Louis Encephalitis

Age of mice	Dilutions of virus					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
3 wks.	5 5 5 S	5 6 6 7	8 15 8 S	9 S S S	—	—
6 wks.	5 6 S 8	S 8 S 8	S S S S	—	—	—
15 wks.	S S S S	S 8 S S	S S S S	—	—	—
3 wks.	4 4 5 5 5 5 7 S	4 5 5 5 6 6 6 6	5 8 8 S S S S S S	—	—	—
6 wks.	5 6 6 10 S S S S	5 7 S 8 8 S S S	—	—	—	—
15 wks.	5 6 10 S S S S S	—	—	—	—	—
2 wks.	4 5 5 5 5	5 6 6 7 8	6 7 8 8 8	8 8 9 9 S	8 8 9 9 S	6 8 10 10 S
3 mos.	6 6 S S	S S S 8	S S 8 8	—	—	—
2 wks.	—	—	5 6 6 6	7 8 8 8	6 8 10 S	8 8 11 S
2 wks.	—	4 4 4 5 5	4 4 4 5 6	7 9 10 11 12	8 8 13 S S	7 8 8 16 S

Distribution of Lesions

Webster and Fite (11, 12) and Smadel and Moore (13) described the histological changes produced by the virus of St. Louis encephalitis in mice inoculated intracerebrally, they emphasized the subpial and perivascular accumulations of mononuclear cells, foci of microglial proliferation about vessels, and beneath the pial membrane and necrosis of neurons Webster and Clow (14) proved that virus instilled intranasally invaded the brain by the olfactory route. The presence of the virus in the olfactory bulbs was demonstrable 48 hours before the occurrence of the earliest lesions in that site

The lesions which we have studied are, for the most part, the perivascular accumulations of cells and the mesodermal-glial response Bodian and Howe (15, 16), studying experimental poliomyelitis in the monkey, demonstrated the feasibility of utilizing these lesions to follow the invasion of the C N.S by neurotropic viruses After intranasal instillation of the virus of poliomyelitis, monkeys killed during the preparalytic stage of the disease showed lesions in the primary olfactory regions, and in the hypothalamic nuclei, the tegmentum of the mid brain and the reticular formation of the hind-brain,—centers con

nected with the olfactory region by the olfactotegmental pathway. Certain centers not on this primary preferential pathway such as the amygdaloid nuclei, parolfactory and septal areas, pyriform cortex, and midline and dorsomedial thalamic nuclei, were also involved. In the late paralytic stage this pattern of distribution was not obscured but lesions were also found associated with the pathway between the motor cortex and the lower centers. In the late paralytic stage of the ascending infections, which followed inoculations into the sciatic nerve, peritoneal cavity or spinal cord, the distributions of lesions were similar to those observed following intranasal instillations, but as a rule lesions were more abundant in the reticular formation and tegmentum and diminished suddenly rostral to the region of Forel's fields. Lesions were absent from the olfactory bulbs, most of the other olfactory centers and the midline thalamic nuclei.

Because of the small size in the mouse brain references to cytoarchitectonic designations of many well known nuclei have been avoided where possible and the observations have been reported in the conventionally used gross descriptive terms. However, it has been convenient to divide the pyriform cortex into retrobulbar, prepyriform, and periamygdalar regions, and to mention the more caudally situated entorhine region from which the hippocampus receives abundant olfactory afferent fibers. The site and extent of each of these regions is given in the cytoarchitectonic atlas of Rose (17). In the present study, the dorsal thalamus has been divided into anterior, medial, and lateral areas. The medial area includes the midline as well as the medial nuclear groups, the lateral area contains the cortical relay nuclei. The anterior nuclei are not mentioned because they appear in relatively few sections. Considerations based on the blood supply of the thalamus of the rodent (Schlesinger, 18) indicate that further subdivision is inadvisable. The nuclear configuration of the tegmentum of the mid-brain and the reticular gray of the hind-brain is described and illustrated by Craigie (19).

Intranasal Series.—Four groups totalling 35 mice 6 to 8 weeks of age received 10^{-3} or 10^{-4} dilutions of virus instilled nasally. The time of appearance of the clinical symptoms varied among the different groups with no constant relation to the dilution of the virus employed. Histologically it was found convenient to divide the series into early and late stages based upon the extent of invasion of the C. N. S.

In the early stage, lesions were localized primarily to the basal olfactory territory of the telencephalon and to centers associated with the olfactotegmental pathway leading from that territory to the mid- and hind brains. Five of the 24 brains removed before clinical signs of disease developed showed such localized lesions, 11 were entirely negative, and in the remainder the lesions were more widely distributed.

Webster and Clow (14) recorded perivascular accumulations of lymphocytes in the olfactory bulb as early as the 3rd day following intranasal instillation of the virus. We have confirmed this observation in one brain that was otherwise negative. In 4 other brains, obtained from animals killed upon the 4th and 5th days following

inoculation there was more extensive invasion of the C.N.S Accumulations of cells were prominent about many vessels in the olfactory bulbs, and occasional foci of mesodermal glial proliferation were observed The retrobulbar and prepyriform regions showed similar lesions The periamygdalar region was involved to a lesser degree, but occasionally slight proliferation of microglia in its plexiform layer was also found At this time lesions also appeared in the tubercula olfactoria septa, and ventral parts of the caudate nuclei The latter appeared to have been invaded from the tubercula olfactoria and pyriform territory Lesions were observed in the preoptic areas in all of these brains but in 2 of the 4 none were encountered caudal to this level In the other 2 brains lesions were also observed in the hypothalamus, the medial areas of the dorsal thalamus, tegmentum of the mid brain and the dorsal part of the reticular gray at the rostral end of the hind brain In addition there was usually a slight involvement of the frontopolar frontal interhemispheric, and temporal parts of the cortex which closely adjoin the basal olfactory territory

An outstanding feature of the early stage was the constancy with which the lesions upon one side were more severe and abundant than upon the other This difference was most obvious in the *pyriform territories and tubercula olfactoria* but also appeared in the caudate nuclei, dorsal thalamus and tegmentum Differences between the two sides of the brain were also encountered in the intraperitoneal series other wise *this result could be attributed to the amount of virus which entered one nostril* as compared with that entering the other

The lesions in the late stage were no longer confined to the primary olfactory centers and those associated with the pathway leading to the tegmentum and reticular formation This more widespread distribution characterized the brains obtained from animals killed at the onset of clinical signs but was also observed in some of the *brains obtained in the preclinical period. In the pyriform territory (Figs 1 and 2)* and tubercula olfactoria the mesodermal-glial response was always more severe than that observed during the early stage this response in the plexiform layer of the pyriform cortex usually diminished in intensity from the retrobulbar toward the entorhine region In 2 specimens of the late stage widespread necrosis of nerve cells had occurred throughout the pyriform territory (Fig 3) but this was not accompanied by an unusually severe mesodermal-glial reaction Lesions were also abundant elsewhere in the fore-brain although parts of the neocortex or one hippocampus sometimes escaped involvement Lesions of the mid-brain occurred in the central gray and tectum as well as in the tegmentum Lesions of the hind brain were either localized to the dorsal part of the reticular gray as observed in specimens in the early stage, or were scattered throughout the reticular formation to the caudal end of the medulla.

Summary—Following intranasal instillation an early stage of distribution of lesions can be recognized in which the primary *olfactory* centers and those associated with the olfactotegmental pathway are principally involved

Intraperitoneal Series—Groups of mice of different ages (2, 3 4 and 6 to 8 weeks) were inoculated intraperitoneally with different concentrations of virus (Table IV) and killed usually in groups of 3, at half day intervals from 2 days following inocula *tion until clinical signs of disease* appeared. Significant enough differences in distri

butions of lesions did not appear between the brains of the 3, 4, and 6 to 8 week age groups to warrant separate presentation Therefore they are reported as a unit followed by the findings in 2-week-old mice

All brains and spinal cords of mice in the 3 to 8 week age group were negative until 3 days after inoculation When 10^{-1} dilution of virus was used, a limited distribution of lesions was found in at least 1 of each group of 3 brains obtained at that time, at intermediate periods ($3\frac{1}{2}$ to 5 days) an increasing number of brains contained lesions, and at 5 to 6 days a majority of them With 10^{-2} and higher dilutions of virus a large majority of the brains including all those observed at the 3 day period were negative Lesions did not appear until later and, compared with corresponding periods where 10^{-1} dilution of virus was used, the distribution of lesions was frequently quite limited

In 2 specimens of the 3 to 8 weeks age group convincing evidence was obtained that ascending invasion of the C N S occurs after intraperitoneal inoculation Le-

TABLE IV

Number of Mice of Different Ages Inoculated Intraperitoneally with Different Concentrations of Virus

Age	Concentration of virus		
	10^{-1}	10^{-2}	Higher dilution
wks			
2	11	14	10
3	11		26
4	20	23	—
6–8	24	—	—

sions were found in the spinal cord and scattered through the reticular formation of the medulla In one brain, the lesions disappeared in the hind-brain at the level of the nucleus of the VIIth nerve, in the other, the tegmentum of the mid-brain was also involved and a group of lesions appeared in one lateral thalamic area and in the corresponding parietotemporal area of the neocortex The lesions in the lateral thalamic area also extended toward the parafascicular nucleus which is situated in the medial thalamic area

The majority of infected brains obtained at early and intermediate periods (10^{-1} dilution of virus) showed lesions which were scattered and mild in the pyriform territories, absent or very slight in the entorhine regions, hippocampi, and olfactory bulbs The spinal cords were not invariably involved but lesions always appeared in the reticular formation at the caudal end of the hind brain Rostrally in the hind brain, they were usually confined to the dorsal reticular gray Lesions appeared in the tegmentum, central gray, and frequently in the tectum of the mid brain and in the lateral and medial areas of the dorsal thalamus The hypothalamus, preoptic areas (Fig 5), and tubercula olfactoria were also involved In the latter region lesions were sometimes severe in contrast to the mild lesions usually observed in the pyriform territories A variable part of the neocortex was frequently involved

In other specimens obtained at the late periods, the spinal cords were always involved and the pyriform territories frequently showed extensive mesodermal-glial proliferation as well as perivascular accumulations of lymphocytes Two of these brains showed widespread necrosis of nerve cells in the pyriform, periamygdalar and entorhine regions of one side. Lesions always appeared in the hippocampi, entorhine regions, and olfactory bulbs, and in the plexiform layer of the bulbs extensive proliferation of mesodermal glia has been observed

Finally in another small but significant group of brains belonging to the 3 to 8 weeks age group, and not restricted to the younger of these mice, the distribution of lesions indicated that invasion had occurred by the olfactory route The lesions in these brains were more advanced and abundant in the olfactory bulbs (Fig 4) and pyriform territories as compared with those observed further caudally the spinal cords were almost always negative.

An isolated cluster of lesions widely separated from an obvious portal of entry occurred in only one brain (4-week-old, 10^{-2} dilution of virus, 4th day) This was observed in the interior of the cerebellum

In 2 week-old mice receiving a 10^{-1} dilution of virus, lesions were distributed throughout the C.N.S as early as 2½ days following inoculation It was impossible to distinguish the portal of entry but the distribution of the lesions did not differ from those observed in older mice in the late stage in such a way as to suggest that the virus had involved the C.N S by the hematogenous route. Using 10^{-2} dilution of virus the lesions did not appear until 3 days following inoculation but likewise involved the entire C N.S although they appeared more scattered than when the 10^{-1} dilution of virus was used. Using still higher dilutions of virus the first appearance of lesions was retarded and a limited distribution similar to that obtained in the mice of the 3 to 8 weeks age group was obtained One mouse killed on the 7th day following inoculation with a 10^{-4} dilution of virus showed a limited distribution of lesions indicating that the virus had entered the brain by the olfactory portal. In another mouse which showed a flaccid paralysis upon the 7th day (10^{-4} dilution of virus), a typical ascending distribution of lesions occurred in which the fore brain was completely negative.

Summary—The results in all age groups indicate that the entire C.N.S may be invaded by a virus which reaches it through the spinal cord and that this method of invasion probably occurs in the majority of mice inoculated intraperitoneally However, a small but significant number of animals, irrespective of age, give decisive evidence of invasion of the C.N.S by the olfactory route Limited distributions of lesions do not occur in mice 2 weeks of age following intraperitoneal inoculation of 10^{-1} or 10^{-2} dilutions of virus, nevertheless they do occur when the virus is inoculated in higher dilutions

DISCUSSION AND SUMMARY

The greatest change in the susceptibility of mice to the virus of St Louis encephalitis inoculated intraperitoneally occurs between the 2nd and 3rd weeks of life To investigate the mechanism of the influence of age on sus-

ceptibility, mice of different ages (2, 3, 4, and 6 to 8 weeks) were killed at intervals following intraperitoneal inoculation and the distribution of the lesions in the C N S was compared with that which resulted from infections via the nasal portal in mice 6 to 8 weeks of age

Following intranasal instillation of the virus, lesions first appeared in the olfactory bulbs and advanced through the pyriform areas and the other fore-brain olfactory centers Before the entorhine region and hippocampus were involved, lesions had already appeared in centers associated directly or indirectly with the olfactotegmental tract The remainder of the C N S was more or less extensively involved in mice showing clinical signs of the disease

In mice 2 weeks of age inoculated intraperitoneally with 10^{-1} dilution of virus, lesions appeared throughout the C N S as early as 2½ days subsequent to inoculation, the virus spread so rapidly that the portal of entry could not be detected The majority of the mice of the same age receiving 10^{-2} to 10^{-5} dilutions of virus and of the older mice receiving 10^{-1} dilution of virus showed intermediate stages in the progression of lesions through the C N S In the latter group this pointed to an ascending involvement in which the virus reached the fore-brain olfactory centers in retrograde fashion, the preoptic areas and tubercula olfactoria were extensively involved before the pyriform areas and olfactory bulbs In another small but significant group of intraperitoneally inoculated mice representing all ages, lesions of restricted distribution occurred in the C N S which appeared in all respects identical with those which followed infection by the nasal route

The significant difference in the susceptibility to infection between mice 2 weeks of age inoculated intraperitoneally and those 3 weeks of age or older inoculated by the same route is not readily explained by changes in the permeability of capillaries to the virus It has been pointed out that in the 2-week-old mice (10^{-1} dilution of virus) the whole C N S was involved as early as 2½ days following intraperitoneal inoculation However, the distribution of lesions did not differ materially from those observed when the C N S was completely involved in older mice killed at longer periods following inoculation Therefore, the conclusion cannot be drawn that virus reaches the C N S of the 2-week-old mice directly through the blood steam while it reaches that of the mice 3 weeks of age and older by axonal pathways leading from the portal of entry Our data strongly support the view that contamination of the nasal mucous membrane and subsequent invasion of the C N S by the olfactory route occurs not uncommonly in mice inoculated by peripheral routes (Sabin and Olitsky, 3), but we have observed no age difference in this respect for the virus of St Louis encephalitis

The C N S of the mouse during the first 2 weeks of life is still undergoing rapid changes leading to a complete neuronal and glial differentiation In a critical discussion of the tectogenetic principle of cortical development Lorente

de Nó (20) has presented evidence concerning the stage of development attained by the entorhine cortex at intervals preceding and after birth He observed that the migration of glial cells from the ependymal layer through the cortical plate began shortly before and was not finished until several days after birth, at 5 days glial cells had already entered the cortex and presented a very mature form, but other cells of the ependymal layer were still commencing their migration Other evidence demonstrated that different kinds of neurones in the entorhine cortex could be recognized in their embryonic form at 12 hours subsequent to birth, at 5 days differentiation was more advanced in the deeper cortical layers but was still incomplete throughout Definite information was not given concerning the age at which the glial and neuronal elements of the whole cortex become completely developed, but presumably it is considerably later than 5 days

Therefore, the hypothesis that the mature C N.S is a less favorable substrate for the growth of neurotropic viruses than the immature one must be considered as a possible explanation for the greater susceptibility of 2 week-old mice to virus inoculated intraperitoneally Reviewing the results obtained following intraperitoneal inoculation with the 10^{-1} dilution of virus it is apparent that only in mice 2 weeks of age did the virus spread so rapidly through the C.N.S that lesions could be detected everywhere as early as 2½ days subsequent to inoculation In certain mice of the 6 to 8 weeks age group a restricted distribution of lesions was encountered at 3 and 3½ days, but it was not until later that the C.N.S became completely involved Also favoring the point of view that tissue of the nervous system may vary in its ability to support the growth of the virus is the fact that certain centers such as the entorhine regions, hippocampi, and neocortex may escape involvement in the mice 3 to 8 weeks of age killed at the early periods following inoculation

On the other hand, the results obtained with the higher dilutions of virus (10^{-4} and 10^{-8}) in 2 week-old mice indicate that whatever difference may exist between the resistance of the immature and the mature nervous substrate must be only relative Protracted incubation periods can occur and quite restricted distributions of lesions remain as late as 7 to 9 days following the inoculation of small amounts of virus This observation, considered with the equal susceptibility of young and old mice to virus inoculated intracerebrally or instilled intranasally makes it seem unlikely that the greater susceptibility of young mice to virus inoculated intraperitoneally depends upon the immaturity of the tissue of the C.N S

All the observations point to the importance of the amount of virus reaching the C.N.S following intraperitoneal inoculation A possible explanation of the difference in susceptibility, as well as the early appearance of widespread lesions in the young animal receiving large amounts of virus, is that a greater amount of the virus inoculated intraperitoneally survives in the young animal

and reaches accessible portals of the C N S The data are consistant with this explanation, as is the recent study of Morgan (21), who drew a parallel between the resistance of young vaccinated and adult normal mice to peripheral inoculation of the active virus of Eastern equine encephalomyelitis The analogy drawn between young vaccinated mice and adults was based on the observation that, although susceptible to virus given by the intracerebral route, the young vaccinated animals, by the 4th day after the beginning of vaccination, resisted large doses of virus given by the intraperitoneal route These results linked with the demonstration of the more rapid immune response of older animals to the virus of Eastern equine encephalomyelitis, as judged by the appearance of demonstrable neutralizing antibody (10), appear to offer an explanation for the greater resistance of older mice to the peripheral inoculation of active virus of Eastern equine encephalomyelitis

The failure of some investigators (22) to demonstrate neutralizing antibody in the serum of mice immunized by the subcutaneous inoculation of active virus of St Louis encephalitis until several weeks following the immunizing inoculation might seem to weaken the evidence for the immune response being involved in the resistance of mice to the peripheral inoculation of the virus of St Louis encephalitis However, recent observations by one of us (23) have shown that neutralizing antibody to the virus of St Louis encephalitis can be demonstrated at least as early as one week (the earliest interval when tested) following the subcutaneous inoculation of mice over 3 weeks of age with active virus

CONCLUSIONS

1 Young mice are more susceptible than older mice to the virus of St Louis encephalitis inoculated intraperitoneally, but with virus inoculated intracerebrally or intranasally, there is no significant age difference in susceptibility The greatest change in the resistance to the virus inoculated intraperitoneally occurs between the 2nd and 3rd weeks of life

2 The distribution of the lesions of St Louis encephalitis in the C N S of young and of old animals following intraperitoneal inoculation indicates that the virus may reach the brain either by the ascending pathway from the spinal cord or by the olfactory pathway irrespective of the age of the animal However the ascending pathway is most frequently concerned

3 The distribution of lesions does not offer evidence that the virus enters the C N S of young animals directly from the blood stream following intraperitoneal inoculation

4 Although widespread lesions occur earlier in the C N S of young mice than in that of older mice inoculated intraperitoneally with large doses of virus, this fact is not satisfactorily explained by assuming the more rapid increase of the virus in the C N S of young animals, since the latter are not more susceptible to virus inoculated directly into the brain

5 The observations can be explained by the hypothesis that a greater amount of virus survives and reaches the portals of the C.N.S in young animals following intraperitoneal inoculation and that this is an important factor in the influence of age on susceptibility to the virus

We are greatly indebted to Mr John Eisenhauer who prepared much of the histological material

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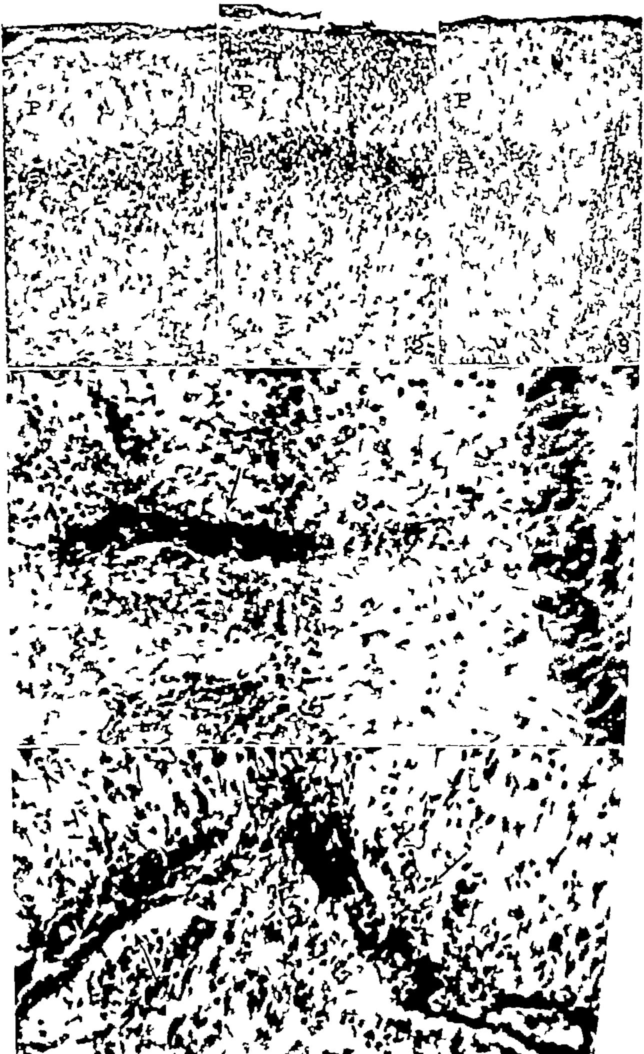
EXPLANATION OF PLATE 4

FIGS 1 and 2 Subpial glial reaction in mice 6 weeks old receiving 10^{-3} dilution of virus by nasal instillation From the cortex of the pyriform lobe *P*, plexiform layer, *S*, layer of superficial pyramids $\times$ 100

FIG 3 Same area as represented in preceding figures Illustrates a massive necrosis of nerve cells that followed nasal instillation of virus in a 6 week-old mouse, layers of superficial (*S*) and deep pyramids have been destroyed This reaction was also observed in animals infected by the peritoneal route $\times$ 100

FIG 4 Olfactory bulb of a 3 week-old mouse that received 10^{-1} dilution of virus intraperitoneally and was killed 3½ days later Note the accumulation of lymphocytes about penetrating vessels (arrow) No lesions were observed in this brain caudal to the preoptic area Scattered vessels of the spinal cord were surrounded by accumulations of lymphocytes $\times$ 250

FIG 5 Accumulation of lymphocytes (arrows) about vessels of the lateral preoptic area of a 6-week-old mouse that received 10^{-1} dilution of virus by the peritoneal route and was killed 4½ days thereafter $\times$ 250



STUDIES ON THE MECHANISM OF IMMUNITY IN TUBERCULOSIS

The Fate of Tubercle Bacilli Ingested by Mononuclear Phagocytes Derived from Normal and Immunized Animals

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Plates 5 and 6

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The mechanism of the increased resistance to reinfection in tuberculosis is still uncertain It has been demonstrated that bacilli of reinfection are either destroyed at once (1), if small numbers penetrate the tissues, or fail to multiply in the immune animal if large numbers invade the body (2), whereas in the normal individual the bacilli, whether many or few at first grow un hindered As to the mechanism of this growth inhibition in the immune animal, it may be said that, while antibodies in low titer develop during the the course of tuberculosis, there is no evidence of specific bacteriolysins acting *in vitro* (3) However, *in vivo*, studies have shown that in the body fluids of the immune animal the growth of tubercle bacilli is much less than that taking place in the body fluids of normal animals (2, 4) Since the bacillus multiplies not only in the tissue fluids but especially within the cytoplasm of the cells of the normal animal (5), the apparent failure of the microorganism to multiply within the phagocytes of the immune animal (6) must be explained

The increased rapidity of the mobilization of mononuclear phagocytes at the site of invasion of the parasite, which characterizes the response of the immune as contrasted with that of the normal animal, has been recently shown to be a function of a heightened physiological activity conferred upon these cells by the tuberculous process (7) This results in an increased rate of cell division on the part of these phagocytes in response to the microorganism in the immune animal Furthermore, this heightened physiological activity of the mononuclear phagocytes of the infected animal expresses itself by an increased phagocytic capacity for a variety of particulate substances such as carbon particles, staphylococci, and collodion particles Tubercle bacilli are also more readily phagocyted *in vitro* by mononuclears derived from actively tuberculous animals than by those obtained from normal animals This is independent of the menstruum of normal or immune serum in which they are suspended and is a property of the cells themselves It is noteworthy that

this property is not significantly increased in animals vaccinated with an avirulent microorganism

Whether the observed failure of bacilli of reinfection to grow within the mononuclears of the tissues of the immune animal is also an expression of the altered physiological activity of these cells is the burden of the present study It is obvious that mere increased phagocytosis of tubercle bacilli by "immune" phagocytes is no explanation for the failure of bacilli to grow within them There is abundant phagocytosis of tubercle bacilli by the mononuclears of the normal animal (8) and it is precisely within the cytoplasm of normal phagocytes that the bacilli multiply

Hanks and Brockenbrough (9) have recently reported that serum leukocyte mixtures from normal, infected, and immunized rabbits fail to show any bacteriocidal action on human tubercle bacilli *in vitro* Rich (10) states that cells derived from immunized animals that ingest tubercle bacilli while growing in tissue culture fail to show a greater inhibitory effect on the multiplication of tubercle bacilli within them than cells derived from normal animals similarly cultivated

Thus *in vitro* methods have failed to duplicate the inhibition of growth of bacilli that can be readily observed to occur in the living tissues of the reinfected animal Since the bacteriostatic action of the body fluids of the immune animal cannot be demonstrated *in vitro*, but can be shown to occur by *in vivo* methods, it appeared possible that by *in vivo* methods one might demonstrate what appears to be the bacteriostatic effect of the cells on the growth of tubercle bacilli in the tissues of the reinfected animal Greene (11), Lucké (12), and others have recently used the anterior chamber of the eye of the living animal as a culture medium for the growth of living tumor cells It appeared possible that this method might also be applied to the problem on hand

All previous studies have indicated that, even with the highest degree of immunity attainable, the bacilli of reinfection are rarely, if ever, completely annihilated at least a few bacilli always remain It is obvious therefore, that quantitative methods must be used in order to answer the question whether within the cytoplasm of cells derived from immune animals tubercle bacilli are inhibited in their growth as compared to that taking place within the cytoplasm of phagocytes derived from normal animals

It appeared desirable at first to test the efficacy of the *in vivo* method for the solution of this question under the most advantageous, rather than the most crucial conditions

Methods and Materials

Normal and immunized rabbits were given a subcutaneous injection of human or bovine type tubercle bacilli suspended in salt solution containing India ink The lymph nodes draining the site of injection, both in the normal and in the immunized

animal, were removed 2 days following the inoculation. Each lymph node was then divided into three portions one was weighed, ground, and cultured, to determine the number of living bacilli it contained, an adjacent portion of each node was prepared for microscopic study, the third weighed portion of the lymph nodes, derived from the normal and the immunized animal, respectively, was cut up into fine particles with sharp scissors, washed several times in Tyrode solution, and injected into each anterior chamber of the eyes of a normal, non tuberculous, albino rabbit previously treated with novocaine The cells derived from the lymph node of the normal animal containing the bacilli and the carbon particles that had been carried to them by the lymph flow from the site of infection were placed in the anterior chamber of one eye The cells derived from the lymph node of the immunized animal with their burden of tubercle bacilli and carbon particles originating at the site of reinfection, were placed in the same amount, in the anterior chamber of the opposite eye of the same albino host. In each instance the chamber fluid was first withdrawn by means of a 26 gauge needle, attached to a tuberculin syringe and inserted tangentially in the anterior chamber through the limbus of the cornea Leaving the needle in place within the chamber, the syringe was removed and another tuberculin syringe containing the desired cell suspension, was attached and the suspension injected into the chamber in 0 1 to 0.2 cc. volumes

The growth of the implanted cells was observed for a period of 10 to 14 days At the end of this time the rabbit host was killed. Both eyeballs were removed and fastened with sterile push pins on sterile wooden squares The fluid from each chamber was withdrawn into a syringe containing a suitable amount of 3 per cent sodium citrate or a heparin anticoagulating solution. This fluid, after microscopic examination, was appropriately diluted and cultured to determine the number of living tubercle bacilli it contained. The cornea was then removed by a circular incision. The entire iris and the ciliary body was then ablated from the underlying lens and vitreous humor, together with any growth which was attached to these structures This tissue was now divided into three portions one was examined microscopically by direct smear, the other was fixed for histological study and the third, constituting the major portion of these structures, was weighed ground, and suspended in suitable amounts of fifteenth molar Na_2HPO_4 and cultured to determine the number of tubercle bacilli which it contained.

Identical experiments were done with bone marrow obtained from normal and immunized animals respectively, that had received intravenously, 2 days previously, 0 1 to 10 mg of human type tubercle bacilli mixed with India ink.

Fate of Tubercle Bacilli Ingested in Vivo within Cells of Normal and Immunized Rabbits

In this test the tubercle bacilli, both human and bovine, had been phagocyted *in vivo* by cells of normal and immunized rabbits in the menstruum of their own body fluids. These normal and "immune" cells with their ingested tubercle bacilli and carbon particles were washed to remove adhering body fluids and transferred into the environment of the anterior chambers of a normal rabbit Since the rabbit hosts were albinos, no pigment was present in

their irides or ciliary bodies and the cells implanted in the chambers could be identified by the carbon particles which they contained. A correlation was made of the growth of these implanted cells on or in the iris and ciliary body of the host rabbit, the bacilli which they contained, as seen in their microscopic sections, and the number of tubercle bacilli cultured from an adjacent portion of these structures. A comparison of the number of bacilli contained in the lymph nodes or in the bone marrow originally implanted in each anterior chamber with the number of bacilli cultured from the corresponding chamber fluid, the iris and ciliary body after 10 to 14 days incubation, gave an estimate of the relative inhibition or multiplication of the bacilli that had taken place in each chamber.

In Table I*a*, are detailed the exact conditions under which each of 7 such experiments was done. The results obtained from these experiments are recorded in Table I*b*.

It will be noted in Table I*b*, in Experiments 4 to 7 inclusive, that the number of bacilli recovered from a unit weight of bone marrow obtained from immunized rabbits that had received an intravenous injection of 0.1 to 10 mg. of human type tubercle bacilli 2 days previously, was invariably greater than that cultured from the marrow of the normal animal similarly inoculated with the same amounts of the same suspension of tubercle bacilli (columns 2 and 6). Since the large numbers of bacilli injected have not been destroyed in the marrow of either animal, the greater numbers cultured from the immunized animal may be ascribed to the possibility that more bacilli had been removed by the bone marrow of the immunized animal from its circulation than had occurred in the normal animal. This would be in accord with the previously demonstrated fact that the phagocytic capacity of mononuclears of tuberculous animals for tubercle bacilli is greater than that of normal animals. The phagocytosis in the tuberculous animal is here further enhanced over that in the normal animal by the immune body fluids of the former.

Ten to 14 days after incubation of these marrows in the anterior chambers of a normal host, the iris and chamber fluid implanted with cells derived from the normal animal contained many more living bacilli than the same structures implanted with "immune" cells, despite the greater original number of bacilli contained in the "immune" implants. In the three remaining experiments, in which lymph node tissue of normal and immunized rabbits, containing either human or highly virulent bovine bacilli drained from subcutaneous foci of primary infection and of reinfection, respectively, was incubated in the anterior chambers of the same normal host, there were again much greater numbers of bacilli in the chamber implanted with cells of normal origin than in that implanted with cells of immune origin. In all of these 7 experiments the multiplication of the bacilli implanted together with normal cells was from 2 to 10 times as much as that of bacilli implanted in association with cells derived

TABLE I

Fate of Tubercle Bacilli Ingested in Vivo within Cells of Normal and Immunized Rabbits Implanted in the Anterior Chamber of Normal Animals

(*a*) *Conditions under Which Experiments Were Performed*

Experiment No	Tissue tested	Dose, type, and route of inoculation of bacilli	Mode of immunization of donor of immune cells and interval, in days between beginning of treatment and time of test	Days of incubation of implanted tissue in anterior chamber
1	Axillary nodes	1.0 mg, human subcutaneously	0 1 mg human bacilli intravenously followed 1 mo later by 0.2 mg and 1 0 mg. of the same strain intracutaneously and subcutaneously respectively 109	11
2	Popliteal nodes	4 0 mg bovine, subcutaneously	0 0001 mg virulent bovine bacilli intravenously 84	14
3	Popliteal nodes	4.0 mg., bovine, subcutaneously	0 0001 mg virulent bovine bacilli intravenously 95	14
4	Bone marrow	2.0 mg human intravenously	Same mode of immunization as in Experiment 1 * 162	14
5	Bone marrow	0 1 mg human intravenously	4 0 mg., human, subcutaneously 38	10
6	Bone marrow	0 4 mg., human intravenously	1 0 mg, human intravenously 38	14
7	Bone marrow	10 0 mg human intravenously	1 0 mg human bacilli intravenously followed 4 mos. later by 0.2 and 1 0 mg of the same strain intracutaneously and subcutaneously respectively 134	14

* An additional 1 0 mg human bacilli given intravenously 15 days before test.

(*b*) *Results Obtained*

Experiment No.	Original No of colonies cultured from 100 mg of cells derived from normal animal	No of colonies cultured from 100 mg of iris implanted with normal cells	No of colonies cultured from 0 1 cc. of chamber fluid implanted with normal cells	Ratio between No of colonies cultured from iris and from cells originally implanted	Original No of colonies cultured from 100 mg cells derived from immunized animal	No. of colonies cultured from 100 mg of iris implanted with "immune" cells	No of colonies cultured from 0 1 cc. of chamber fluid implanted with "immune" cells	Ratio between No of colonies cultured from iris and from cells originally implanted
1	5	202	1	40 40	6	28	0	4 66
2	5 360*	3 200	—	0 60	5,160*	1 700	55	0 33
3	39 000*	78 700	67 400	2 02	15 000*	4 300	7 100	0 28
4	872	41 800	2 730	47 93	1 118	14 900	64	13 33
5	960	20	0	0 02	1 790	20	0	0 01
6	9 470	8 300	672	0 88	25 740	1 900	408	0 07
7	59 800	66 000	2 950	1 10	91 800	16 100	1 450	0 17

* Ravenel (bovine)

from an immune animal, both implants growing in identical environment in the two chambers of the same normal rabbit host (Compare column 3 with 7 and 5 with 9)

Microscopic examination of the iris implanted with cells of normal animals showed variable accumulations of intact, large mononuclears growing diffusely or in nodular formation on the surface or in the stroma of the iris. In the cytoplasm of these cells, which was often greatly developed and composed of large vacuoles, there were found large numbers of long, deeply and uniformly stained tubercle bacilli (Fig. 1). These were frequently arranged in the form of skeins or packets of parallel rods. In the cytoplasm of the same cells were seen variable amounts of carbon particles in varying degrees of dispersion. These particles as well as the bacilli were at times found in the form of a circle about the centrosphere of the cells. The mononuclears were intermixed with variable numbers of polymorphonuclear leukocytes in varying degree of preservation. At the periphery of these mononuclear growths mitotic figures were frequently encountered. There was a variable degree of accumulation of mononuclears without carbon particles in the stroma of the iris, apparently of host origin, *which seemed to multiply in proportion to the multiplication of the bacilli in* the implanted cells.

By contrast, in the opposite iris of the same host, implanted with cells of the immunized animal, the multiplication of the implanted cells was much less pronounced. The carbon particles within the cells were often more numerous and less dispersed. In these cells the bacilli were usually much fewer than in the cytoplasm of the implanted cells of normal origin. They were usually very short, faintly stained and often beaded (Fig. 2). The cytoplasm of the implanted cells was characteristically finely vacuolated and, occasionally, typical mature epithelioid cells were seen. There was usually little polymorphonuclear infiltration of the explanted cells. The multiplication of the host cells was usually slight corresponding with the slight multiplication of the bacilli in the implanted "immune" cells.

On the iris implanted with bone marrow the growths frequently retained their original, natural structure with preservation of the fat spaces. However, in these explants the hemopoietic cells and most of the polymorphonuclears degenerated. Only the reticular cells with their burden of carbon particles and bacilli remained (Fig. 2). In the iris implanted with lymph node tissue, plasma cells were encountered, presumably derived from the implanted lymphocytes.

By far the greatest number of bacilli seen in the chambers were within carbon-bearing, intact, living cells both in the iris and in the chamber fluid. Occasionally free, extracellular bacilli were encountered and some bacilli were seen in mononuclears not containing carbon. Most of the implanted cells whether derived from normal or immunized animals appeared well preserved and alive. Occasionally, however, necrobiotic cell collections were seen on the surface of the iris implanted with cells of both normal and immunized animals.

It is clear therefore that, while the bacilli cultured from the chamber fluid and the iris did not represent solely those contained within the implanted cells, there is no question but that most of the bacilli cultured were contained within the implanted cells Therefore, it can be said with a reasonable degree of certainty that cells of immunized animals, that had ingested tubercle bacilli *in vivo* and had grown in the environment of the chamber fluid of a normal rabbit, greatly inhibited the multiplication of the microorganisms contained within them, as shown by comparison with the growth of similar bacilli ingested *in vivo* within cells of normal animals and incubated in the chamber fluid of the opposite eye of the same rabbit host. Whether the inhibitory effect of the "immune" cells on the growth of bacilli present in them is due to the properties of these cells themselves cannot be decided from this experiment For it is conceivable that the greater inhibition of bacillary growth in these cells as compared with that in normal cells may be due to the film of immune body fluids that had coated the bacilli before their ingestion within the "immune" cells, for the bacilli that were contained within the normal cells were coated with normal body fluids before their phagocytosis. However it is plain from this experiment that no renewal of immune body fluids is necessary for the cells of immunized animals to continue to inhibit the growth of tubercle bacilli within them for 2 weeks after their transfer into the environment of a normal rabbit host

Fate of Tubercle Bacilli Ingested in Vitro within Cells of Normal and Immunized Rabbits

In order to ascertain the rôle of the cells themselves and that of the immune body fluids of the immunized animal in the inhibition of growth of tubercle bacilli within them the ingestion of the microorganism must take place *in vitro* in the presence of both normal and immune serum, respectively It must be first determined whether cells of normal origin will also inhibit the growth of tubercle bacilli within them if the phagocytosis of the bacteria takes place in a menstruum of immune serum It must next be disclosed whether cells of immune origin will continue to inhibit the growth of the bacilli when the latter are ingested in the presence of normal serum Finally, the relative inhibitory effect on the growth of tubercle bacilli must be estimated when cells of normal and immune origin both phagocyte the bacilli in a medium of immune serum. In all these three experiments it would be desirable to grow the cells with their ingested bacilli in the same type of body fluid in which the phagocytosis had taken place

The following procedure was carried out. Normal or immunized rabbits received an intrapleural injection of about 15 cc. of a sterile 30 per cent acacia solution (13) containing 4.25 per cent sodium chloride. Two such preparations were employed, Lilly's and Endo's Both are used clinically for intravenous injection after fivefold

dilution with distilled water The rabbits received this material undiluted Four to 5 days later the sterile exudate was withdrawn from each rabbit in a syringe containing a suitable amount of 0 4 per cent sodium citrate in 0 85 per cent sodium chloride (8) Both exudates were now centrifuged simultaneously at low speed for 4 to 6 minutes The supernatant fluid was pipetted off and the cells were gently washed in 30 cc. of the citrated salt solution just mentioned, and again centrifuged This washing was repeated three times in order to remove the body fluids in which the exudate cells were contained The final cell sediment was then suspended in about 2 cc. of the citrated salt solution Total and differential cell counts were made on each cell suspension Both cell suspensions were then adjusted by proper dilution so as to contain the same number of mononuclears per cubic millimeter The concentration of mononuclears in the various exudates varied to some extent However, in the majority of instances, it constituted well over 75 per cent of the cells The polymorphonuclears and the lymphocytes could not be equalized

To 0 5 cc of each cell suspension was added the same volume of fresh undiluted serum derived from the heart's blood of either the normal or the immunized donor of the exudate cells The amount of serum added to the cells varied from 0 3 to 1 0 cc in the different experiments To each of these serum leukocyte mixtures was added the same amount of the same suspension of human type tubercle bacilli (P 15 B) containing India ink. The tubes were stoppered with sterilized corks coated with melted paraffine and rotated in the Robertson machine (8) in an incubator for 30 minutes at 37°C to permit the phagocytosis of the tubercle bacilli and the carbon particles At the end of this time the tubes were plunged into ice water to arrest phagocytosis and lightly centrifuged for 2 to 4 minutes The supernatant fluid was removed as completely as possible in order to free the cell mixtures from unphagocyted bacilli present therein The number of living unphagocyted tubercle bacilli present in the supernatant fluid was determined by culture

To each cell sediment was now added 0 5 cc of the same fresh normal or immune serum originally present in each phagocytic mixture The chamber fluid from one eye was then withdrawn and one portion of the gently but thoroughly stirred cells with their proper serum fluid was injected into the emptied chamber, as previously described The other cell suspension, containing the cell and serum combination to which the first was to be compared, was similarly injected into the opposite empty anterior chamber of the same albino rabbit host From the remaining portions of each cell mixture smears were made on specially cleaned slides to determine the degree of phagocytosis of tubercle bacilli and carbon particles that had taken place *in vitro* From the same portions of each cell mixture proper dilutions in fifteenth molar Na_2HPO_4 were prepared and cultured on modified Lowenstein medium (14) to determine the number of living tubercle bacilli present in each cell mixture at the time of implantation into each anterior chamber

The cells were thus incubated in the anterior chambers for 14 to 20 days At the end of this time the number of bacilli present in each chamber fluid and in each iris was again determined by culture as outlined above This was correlated with microscopic studies of the same tissues As in the first experiment the implanted cells could be identified by their contained carbon particles A comparison of the number of bacilli originally present in the cells implanted in each chamber with numbers

cultured from the corresponding chamber fluid and iris with its growing implanted tubercle bacilli-bearing cells after incubation, indicated the fate of the bacilli in the cytoplasm of each cell type. Since the chamber fluid was removed before the implantation of the cells and since each cell mixture thus introduced into each chamber was suspended in the same fresh serum in which the *in vitro* phagocytosis of the bacilli had taken place, it is clear that the implanted cells were exposed, for some time at least, to the same fluid medium in which the *in vitro* phagocytosis of the bacilli had occurred.

Fate of Tubercle Bacilli Ingested in Vitro by Cells Derived from Normal Animals in the Presence of Normal and Immune Serum.—As stated above, in evaluating the rôle of the cells themselves and that of the immune body fluids in the inhibition of growth of tubercle bacilli in the cells of the reinfected animal, it must first be determined whether cells of normal origin will also inhibit the growth of the microorganism within them if their phagocytosis and growth takes place in a medium of immune serum. In Table II*b* are presented the results of 6 experiments in which the growth of tubercle bacilli ingested by normal cells in the presence of normal serum is compared to that of bacilli ingested by the same cells but in a medium of immune serum. The experiments were executed as just outlined and the detailed conditions under which these experiments were performed are recorded in Table II*a*.

Column 4 gives the mode of immunization of the donors of immune serum. It will be noted in columns 5 and 6 that the number of mononuclears per cubic millimeter in each of the phagocytic mixtures greatly exceeded the number of individual tubercle bacilli estimated to be present in the same volume. This estimate was based on the weight of bacilli suspended in a given volume of the phagocytic mixture in question. It was assumed that there were approximately 100,000,000 individual bacteria in 1 mg. of pure culture.

In the majority of instances the cell suspensions contained more than 85 per cent mononuclears. There was no constant increment in the phagocytosis of tubercle bacilli or carbon particles *in vitro* by the normal cells when their ingestion occurred in the presence of immune serum as compared with that taking place in normal serum. (These data are omitted from the table to save space.) In the majority of instances after phagocytosis fewer tubercle bacilli remained in the supernatant fluid of the mixture containing immune serum than in that containing normal serum. The difference however was slight and, in all but one instance, within the range of error of the experiment.

In Table II*b*, columns 2 and 6, it can be seen that there was no considerable, constant difference in the number of living bacilli present in the cells after ingestion of the microorganism *in vitro* in either serum at the time of their respective implantation in each chamber. After 14 to 15 days incubation of these normal cells in the chamber of each eye of the same rabbit there was no constant difference in the relative multiplication of the bacilli within the implanted cells in or on the iris, whether the original ingestion of the micro-

TABLE II

Fate of Tubercle Bacilli Ingested in Vitro within Cells Derived from Normal Rabbits in the Presence of Normal and Immune Serum, Planted in the Anterior Chamber of Normal Animals

(a) Conditions under Which Experiments Were Performed

Experiment No.	Inflammatory irritant	Interval between introduction of irritant and withdrawal of exudate	Mode of immunization of donor of immune serum and interval in days between beginning of treatment and withdrawal of serum	No. of mononuclears per c. mm. in phagocytic mixture	Estimated No. of tubercle bacilli per c. mm. in phagocytic mixture	No. of colonies in 100 c. mm. of supernatant fluid of phagocytic mixture containing		Incubation in anterior chamber
						Normal serum	Immune serum	
		days						*days*
1	Lilly's acacia and glycerol broth	4	0.0001 mg. Ravenel intravenously, 116	41,000	3,600	1,040	700	14
2	Endo's acacia	4	Progressive bovine tuberculosis acquired by contact, 120*	29,000	1,500	3,520	4,630	14
3	Endo's acacia	4	0.1 mg. human bacilli intravenously followed 1 mo. later by 0.2 and 1.0 mg. of the same strain intracutaneously and subcutaneously respectively, 47	—	1,500	3,580	2,100	14
4	Endo's acacia	4	Same mode of immunization as in Experiment 3, 53	24,000	1,400	26	13	14
5	Endo's acacia	4	Same mode of immunization as in Experiment 3, 59	21,000	1,500	53	53	15
6	Endo's acacia	5	Same mode of immunization as in Experiment 3, 108‡	11,000	2,000	14,300	2,600	14

* Animal received 0.01 mg. bovine type bacilli 6 days before withdrawal of serum

‡ Animal received 0.001 mg. bovine type bacilli 28 days before withdrawal of serum

(b) Results Obtained

Experiment No.	Original No. of colonies cultured from 100 mg. of cells phagocyting tubercle bacilli in the presence of normal serum	No. of colonies cultured from 100 mg. of iris implanted with cells phagocyting tubercle bacilli in the presence of normal serum	No. of colonies cultured from 0.1 cc. of chamber fluid implanted with cells phagocyting tubercle bacilli in the presence of normal serum	Ratio between the No. of colonies in implanted iris and in originally implanted cells	No. of colonies cultured from 100 mg. of cells phagocyting tubercle bacilli in the presence of immune serum	No. of colonies cultured from 100 mg. of iris implanted with normal cells phagocyting tubercle bacilli in the presence of immune serum	No. of colonies cultured from 0.1 cc. of chamber fluid implanted with normal cells phagocyting tubercle bacilli in the presence of immune serum	Ratio between the No. of colonies cultured from implanted iris and from originally implanted cells
1	233,000	240,000	860	1.03	380,000	113,000	430	0.30
2	108,000	10,300	830	0.09	113,000	29,000	570	0.25
3	66,000	20,000	1,400	0.30	57,300	3,600	2,930	0.06
4	1,000	860	10	0.86	2,600	600	53	0.23
5	3,600	2,300	13	0.64	2,600	1,630	10	0.63
6	113,000	39,300	3,300	0.35	103,000	73,000	800	0.71

organism had taken place in a medium of normal or immune serum Thus in Experiments 1, 3, and 4 the growth in the cells was 3 to 5 times less in the presence of immune than in the presence of normal serum (columns 5 and 9) In Experiment 5 the growth was the same in both and, in Experiments 2 and 6, the growth in immune serum was 2 to 3 times greater than that in normal serum (columns 5 and 9) Similar observations were made on the chamber fluid

Microscopically there was usually little difference in the growth of the implanted cells and their contained bacilli in either iris Their character was essentially the same as that described above for the growth of transplanted normal bone marrow or lymph node tissue that had ingested tubercle bacilli and carbon particles *in vitro* In addition there was found in both chambers a fibrinous matrix attached to the iris in the meshes of which mononuclears bearing carbon and tubercle bacilli were present in diffuse or nodular growths intermixed with variable numbers of polymorphonuclears Irrespective of whether the cells had originally ingested the bacilli in a menstruum of normal or immune serum, there was usually little difference in the number, size, shape, or staining characteristics of the bacilli within the implanted growing cells In some instances, however, the number of bacilli visible within the cells that had ingested the microorganism in the presence of immune serum was less (Figs 3 and 4)

It is clear that immune serum did not impart a constant inhibitory effect on the growth of tubercle bacilli within normal mononuclears when the ingestion of the microorganism took place in immune serum The presence, for some indeterminate time of immune serum in the medium of growth of these normal phagocytes also failed to endow them with constant bacteriostatic properties

Fate of Tubercle Bacilli Ingested in Vitro by Cells Derived from Normal and Immunized Animals in the Presence of Normal Serum —If the failure of the bacilli of reinfection to proliferate within the mononuclears of the tissues of the immunized animal is largely a property of the cells themselves it would follow that cells of immune origin that had ingested the microorganism in normal serum should also inhibit the growth of the phagocyted bacteria when subsequently grown on normal body fluids

Accordingly the growth of tubercle bacilli ingested by cells of immunized rabbits in the presence of normal serum was compared to that of cells from normal animals that had ingested the microorganism in the same medium These normal phagocytes that had ingested the bacilli and the carbon particles *in vitro*, suspended in normal serum, were planted in the emptied anterior chamber of one eye The ' immune ' phagocytes, with their similarly ingested carbon particles and bacilli, suspended in the same normal serum, were implanted in the emptied anterior chamber of the other eye of the same albino rabbit host. All the other measures involved were carried out as previously outlined

In Table III*a* are given the conditions under which 5 such experiments were carried out It may be mentioned that the cells and sera used in 5 of the preceding experiments were derived from the same animals as the cells and sera of the present 5 experiments The original concentration of mononuclears in the pleural exudates of the normal animals varied between 30 1 to 89 5 per cent, that of the immune rabbits ranged between 63 5 and 95 0 per cent Needless to say the percentage of mononuclears of both cell types was equalized before utilization for phagocytosis The average phagocytosis of tubercle bacilli and carbon particles by cells of immune origin was greater than that of mononuclears of normal origin, as in previous experiments However the number of living bacilli remaining in the supernatant fluid of the phagocytic mixture containing "immune" cells did not differ from that remaining in the supernatant fluid of the normal cell mixtures Apparently the difference in the degree of phagocytosis between the normal and immune cells was insufficient to be detected by the number of bacilli remaining in their menstrua It must be emphasized that these experiments were not set up for the purpose of determining the degree of phagocytosis For this latter purpose the bacilli in the menstruum were too few, chance meeting of phagocyte and particle played too much of a rôle The aim was to present so few bacilli for phagocytosis that even the normal cells would take up the bacilli in approximately the same numbers as the immune cells

As seen in columns 2 and 6 of Table III*b* this goal was actually achieved in 4 out of the 5 experiments For in these tests the number of viable bacilli in the normal phagocytic cells before implantation did not differ significantly from that contained in the "immune" cells In these same experiments, the multiplication or rather the survival of the bacilli in the iris of the normal rabbit implanted with "immune" cells was from 5 to 12 times less than that of the iris implanted with normal cells, despite the fact that there was no immune serum in the medium in which either the ingestion of the bacilli or the growth of the phagocytic cells had taken place Likewise in the majority of the chamber fluids implanted with "immune" cells there were much fewer bacilli after 14 to 15 days incubation than in the chamber fluid planted with normal cells The single exception was Experiment 3 where the survival of the bacilli in the "immune" cells was greater than that in the normal phagocytes It is interesting to note in this connection, that the normal and immunized donors of the cells of this experiment were subsequently infected with virulent tubercle bacilli It was found that the normal rabbit showed greater resistance to tuberculosis than the immunized animal The normal animal was a member of a naturally resistant family (15)

Microscopic examination of the growths of the implanted cells confirmed the observations previously detailed The growth of the bacilli in the cells of

TABLE III

Fate of Tubercle Bacilli Ingested in Vitro within Cells Derived from Normal and Immunized Rabbits in the Presence of Normal Serum Planted in the Anterior Chamber of Normal Animals

(*a*) *Conditions under Which Experiments Were Performed*

Experiment No.	Inflammatory irritant	Interval between introduction of irritant and withdrawal of exudate	Mode of immunization of donor of "immune" cells and serum and interval, in days, between beginning of treatment and withdrawal of serum and cells	No. of mononuclears per c mm in phagocytic mixture	Estimated number of tubercle bacilli per c. mm. in phagocytic mixture	Number of colonies in 100 c. mm. of supernatant fluid of phagocytic mixture containing: Normal cells	"Immune" cells	Incubation in anterior chamber
		days						*days*
1	Endo s acacia	4	Progressive bovine tuberculosis acquired by contact 120*	29 000	1 500	1 310	1 880	14
2	Endo s acacia	4	0 1 mg human bacilli intravenously followed 1 mo later by 0.2 and 1 0 mg of the same strain intracutaneously and subcutaneously, respectively 47	—	1 500	4 540	3 010	14
3	Endo s acacia	5	Same mode of immunization as in Experiment 2 108‡	11 000	2 100	7 000	8 600	14
4	Endo s acacia	4	Same mode of immunization as in Experiment 2, 53	24 000	1 400	90	90	14
5	Endo s acacia	4	Same mode of immunization as in Experiment 2 59	21 000	1 500	50	30	15

* Animal received 0 01 mg bovine type bacilli 6 days before withdrawal of serum and cells.

‡ Animal received 0 001 mg bovine type bacilli 28 days before withdrawal of serum and cells.

(*b*) *Results Obtained*

Experiment No	Original No. of colonies cultured from 100 mg. of normal cells phagocyting tubercle bacilli	No of colonies cultured from 100 mg. of iris implanted with normal cells	No. of colonies cultured from 0.1 cc. of chamber fluid implanted with normal cells	Ratio between the No. of colonies in implanted iris and in originally implanted cells	Original No of colonies cultured from 100 mg. of "immune" cells phagocyting tubercle bacilli	No. of colonies cultured from 100 mg. of iris implanted with "immune" cells	No. of colonies cultured from 0.1 cc. of chamber fluid implanted with "immune" cells	Ratio between the No. of colonies in implanted iris and in originally implanted cells
1	122 000	26 000	1 530	0 21	111 000	5 000	360	0 04
2	56 000	7 300	4 100	0 13	65 000	860	130	0 013
3	153 000	2 000	0	0 013	80 000	5 300	130	0 066
4	2 300	2 300	26	1 00	1 600	33	0	0 02
5	3 000	166	0	0 05	2 600	10	16	0 004

normal origin was greater in 4 out of the 5 experiments than that in the implanted cells of immune origin In some of these the difference was very great.

The normal cells actually contained skeins of tubercle bacilli, deeply stained and long (Fig 5), while in the "immune" cells they were difficult to find and those seen were very short, poorly stained, and beaded (Fig 6) Furthermore the cytoplasm of the normal cells consisted of large irregular vacuoles That of the "immune" cells was much finer and more regular Typical mature epithelioid cells with differentiation of the cytoplasm into an external dark periphery and an internal lighter zone were also seen in the "immune" cells growing in or on the iris of the normal host

It is plain therefore that cells obtained from an immunized animal, washed free of their immune body fluids, which had ingested tubercle bacilli *in vitro* in the presence of normal serum and had been planted and grown *in vivo* in the environment of a normal animal bathed either in the normal serum introduced into the chamber or in the natural chamber fluid, inhibited the growth of tubercle bacilli within their cytoplasm, an average of 10 times more effectively than cells of normal animals that had ingested the microorganism and had grown under identical conditions This clearly indicates that the inhibition of growth of tubercle bacilli within the cells of the reinfected animal is a property chiefly of the phagocytic cells themselves and that immune body fluids and the organ environment of the immune animal are not essential for this function

Fate of Tubercle Bacilli Ingested in Vitro by Cells Derived from Normal and Immunized Animals in the Presence of Immune Serum —It was found above that, while immune serum does not constantly impart a significant inhibitory effect on the growth of tubercle bacilli ingested by cells of normal origin, the immune sera of some inmmuized animals did apparently exercise such an effect, to some degree It appeared desirable, therefore, to test further the rôle of the cells and sera by determining the relative inhibitory properties of both agents acting simultaneously Accordingly the growth of tubercle bacilli ingested by cells of normal animals in the presence of immune serum was compared to that of bacilli ingested by cells of immune origin in the same medium

It will be noted in column 4 of Table IV *a*, which details the conditions under which this series of experiments was performed, that all the donors of the "immune" cells and the immune sera used in this series had undergone progressive tuberculosis caused by the highly virulent bovine bacillus, Ravenel At the time when these sera and cells were obtained, this tuberculosis had affected the donors for 44 days to one year This was done in order to elicit a high immune state in these donors

As in the previous series, the average phagocytosis of tubercle bacilli and carbon particles by the "immune" cells was greater than that by the normal cells However, here too there was no constant difference in the number of

TABLE IV

Fate of Tubercle Bacilli Ingested in Vitro within Cells Derived from Normal and Immunized Rabbits in the Presence of Immune Serum Planted in the Anterior Chamber of Normal Animals

(*a*) *Conditions under Which Experiments Were Performed*

Experiment No	Inflammatory irritant	Interval between introduction of irritant and withdrawal of exudate	Mode of immunization of donor of "immune" cells and immune serum and interval, in days, between beginning of treatment and withdrawal of cells and serum	No. of mononuclears per c mm in phagocytic mixture	Estimated No of tubercle bacilli per c. mm. in phagocytic mixture	No. of colonies cultured from 100 c. mm. of supernatant fluid of phagocytic mixture containing: Normal cells	"Immune" cells	Incubation in anterior chamber
		days						*days*
1	Lilly's acacia and glycerine broth	4	0 001 mg Ravenel intratracheally 44	58 000	590	500	130	14
2	Same as in Experiment 1	4	Progressive bovine tuberculosis 1 yr	23,000	140	530	430	20
3	Lilly's acacia	2*	0 001 mg Ravenel intratracheally, 71‡	—	140	250	706	14
4	Endo s acacia	4	0 05 mg. human type intravenously and 0 001 mg Ravenel, by the same route 59 days later the serum and the exudate were withdrawn 58 days after the last injection 117	52 000	1 000	170	940	14
5	Endo s acacia	5	Same procedure as in Experiment 4 the serum and the exudate were withdrawn 70 days after the last injection 129	21 000	1 000	9 600	9 100	15
6	Endo s acacia	4	Same procedure as in Experiment 4 the serum and the exudate were withdrawn 78 days after the last injection 137	100 000	1,000	360	120	14

* The exudate from the corresponding normal rabbit was withdrawn 4 days after injection of the irritant.

‡ This rabbit died with tuberculosis and snuffles pneumonia 2 days after the serum and exudate were obtained

(*b*) *Results Obtained*

Experiment No.	Original No. of colonies cultured from 100 mg. of normal cells phagocyting tubercle bacilli in the presence of immune serum	No. of colonies cultured from 100 mg. of iris implanted with normal cells phagocyting tubercle bacilli in the presence of immune serum	No. of colonies cultured from 0.1 cc. of chamber fluid implanted with normal cells phagocyting tubercle bacilli in the presence of immune serum	Ratio between the No. of colonies in implanted iris and in originally implanted cells	No. of colonies cultured from 100 mg. "immune" cells phagocyting tubercle bacilli in the presence of immune serum	No. of colonies cultured from 100 mg. of iris implanted with "immune" cells phagocyting tubercle bacilli in the presence of immune serum	No. of colonies cultured from 0 1 cc. of chamber fluid implanted with "immune" cells phagocyting tubercle bacilli in the presence of immune serum	Ratio between the No of colonies cultured from implanted iris and from originally implanted cells
1	28 000	22 000	160	0 80	31 000	2 300	16	0 07
2	8 800	15 100	500	1 70	8 600	2 400	230	0 28
3	35 000	3 000	126	0 09	60 000	34 000	1 440	0 57*
4	19 000	63 000	440	3 31	29 000	4 700	100	0 16
5	137 000	209 000	1 800	1 52	120 000	19 000	240	0 16
6	93 000	810 000	1 370	8 71	93 000	5 300	140	0 057

viable bacilli remaining in the supernatant fluid of either cell mixture after phagocytosis. Nor was there any significant difference in the number of living bacilli present in the normal and "immune" cells after phagocytosis in the same immune serum at the time of implantation of these cells in each chamber (Table IV *b*, columns 2 and 6.) These normal and immune phagocytes, each containing approximately the same number of living bacilli and suspended in the same immune serum, were introduced into each emptied anterior chamber respectively of the same normal albino rabbit host.

Fourteen to 20 days later the growth of the bacilli in the normal cells, proliferating in a medium which, for a time at least, contained immune serum was, in 5 out of the 6 experiments, from 6 to 150 times as great as in cells derived from immume animals that had phagocyted the bacilli in the same immune serum and had grown in the same medium. Compare column 3 with 7 and 5 with 9. Likewise the chamber fluid implanted with cells of normal origin contained from 2 to 10 times as many living bacilli in these 5 experiments as the corresponding chamber fluid implanted with "immune" cells. In only one experiment, No. 3, were the results reversed. The donor of the "immune" cells and immune serum for this experiment had extensive snuffles pneumonia superadded upon his tuberculosis. This animal died 2 days after removal of its cells and serum for the test. It is possible that the intercurrent disease affected the immune state of this rabbit's cells and serum.

Microscopic examination of the irides with their implanted, carbon-bearing cells confirmed the cultural results and showed the same differences in the character of the growth of the two cell types and their contained bacilli as had previously been described in those experiments where cells of normal and immune origin were compared. These differences are illustrated in Figs. 7 and 8. The large numbers of the long tubercle bacilli in the cells of normal origin are contrasted with the single, very short bacillus seen in cells of immune origin. It is noteworthy that the carbon particles are in large masses in the cells of immune origin. They are much fewer and more dispersed in the cells of normal origin. Apparently the normal cells had greatly multiplied together with the multiplication of their contained bacilli and subdivided the burden of carbon, originally ingested *in vitro*, among the daughter cells. The "immune" cells, in which the bacilli not only failed to multiply but, as the cultures indicated, had been greatly reduced in number, proliferated but slightly and hence did not reduce the original carbon present in each cell at the time of implantation. Furthermore the normal mononuclears were intermixed with large numbers of polymorphonuclears. Many of the former had undergone injury from the large numbers of bacilli they contained and chemotactically attracted the granulocytes from the host. The "immune" cell growths on the other hand were not intermixed with polymorphonuclears. They were not injured,

as the bacilli had not grown in their cytoplasm, and hence exerted no chemotactic effect on the polymorphonuclears of the host's vessels

It is thus again clearly shown that the inhibition of growth of tubercle bacilli by cells of the immune animal is a function of the cells themselves and that immune serum cannot impart to normal cells an equal inhibitory property for the multiplication of tubercle bacilli within their cytoplasm

Essentially similar results were obtained in a few experiments with guinea pig cells and sera, using normal albino guinea pigs as hosts for the normal and immune cells that had phagocyted tubercle bacilli *in vitro* in normal or immune sera

SUMMARY AND DISCUSSION

An endeavor was made to determine whether phagocytic mononuclears of immunized animals, removed from the influence of the organs in which they are naturally situated, will inhibit the growth of tubercle bacilli in their cytoplasm more effectively than similar cells obtained from normal animals It was also undertaken to ascertain to what extent the inhibition of growth of tubercle bacilli in the immunized animal is due to the cells themselves and to what degree it is accounted for by the immune body fluids which bathe them. To answer these questions the anterior chambers of normal albino rabbits were used as *in vivo* incubators One anterior chamber was host to one type of cell or cell-serum mixture, while the other anterior chamber of the same rabbit was host to a cell type or a cell-serum mixture to which the first was to be compared The fate of the bacilli in each cell type and in each fluid menstruum was culturally determined by comparing the number of living bacilli present in the cells originally implanted with the number of bacilli that were contained in these cells after they had grown in the chambers The implanted cells were identified by carbon particles that had been ingested by these cells at the same time that the bacilli were phagocyted

It was found that cells of immunized animals, which had phagocyted tubercle bacilli *in vivo* in lymph nodes or bone marrow and had been transferred 2 days later into the environment of the chamber fluid of a normal rabbit, inhibited the growth of the microorganism within their living cytoplasm for 2 weeks in the absence of immune body fluids Similarly transferred normal cells, under these conditions, permitted the bacilli to grow within them to a much greater extent. To what degree the inhibition of growth in these 'immune' cells was due to the immune body fluids which had formed a film about the bacteria before their phagocytosis or to the mere sojourn of the bacilli in these phagocytes in the body of the immune animal for 2 days before their transplantation to a normal environment could not be determined by these experiments

To answer the latter question exudative mononuclears derived from normal

and immunized animals, washed free of their body fluids, were permitted to ingest tubercle bacilli and carbon particles *in vitro* in the serum of the normal or of the immunized donor of cells Before incubating these phagocytes in the anterior chambers, the supernatant fluid of each phagocytic mixture was replaced by the same fresh serum in which the phagocytosis had taken place

The presence of antibodies against the tubercle bacillus in the immune sera was not determined It is certain however that in the tissues of such donors of "immune" cells and sera bacilli of reinfection fail to multiply, as had been abundantly demonstrated It is not known how long the immune or normal serum introduced into the chambers with the phagocytic cells persisted there It cannot be said with certainty therefore that implanted cells grew for the entire period of their sojourn in the chamber in the serum introduced with the cells However, there is presumptive evidence to indicate that such introduced fluids remained in the chamber for a considerable time It has been demonstrated by Seegal and Seegal (16) that protein antigens introduced into one anterior chamber sensitize the corresponding eye but not the opposite eye of the same rabbit If proteins introduced into one chamber readily leave it there is no explanation for this phenomenon Under these conditions, it was found that "immune" cells that had ingested tubercle bacilli *in vitro* in the presence of immune serum inhibited the growth of tubercle bacilli in their cytoplasm to a much greater extent than normal cells that had phagocyted the bacteria in the same medium Furthermore, the absence of immune serum in the medium of phagocytosis and growth of cells obtained from immunized animals did not rob them of their greater bacteriostatic properties on the growth of tubercle bacilli within them Nor did the presence of immune serum during phagocytosis impart to cells of normal origin a significant and constant bacteriostatic effect

It is clear therefore that "immune" phagocytic cells possess in themselves, apart from the immune body fluids, a greater bacteriostatic property on the growth of tubercle bacilli within them than do normal cells This demonstration of the inherent bacteriostatic property of the "immune" cells is in harmony with the previously demonstrated increased capacity for phagocytosis of tubercle bacilli possessed by cells of actively tuberculous rabbits, which is also independent of the medium in which the phagocytosis takes place It is significant that this enhancement of phagocytosis parallels the immunity of the possessor of such cells Treatment of rabbits with tubercle bacilli of low virulence confers little immunity to the infection and little or no enhancement of the inherent phagocytic capacity of their cells for tubercle bacilli (7)

The demonstration by Moen and Swift (17) that mononuclears derived from animals sensitive to tuberculin retain that property even after several transplants in tissue culture, shows that allergy, the development of which is so

regularly associated with immunity, is also a property inherent in the cells themselves

These results also support the conclusion drawn from many years' study on the fate of tubercle bacilli and the associated cellular responses in normal and immunized animals, namely, that the destruction or inhibition of multiplication of tubercle bacilli in the reinfected animals rests chiefly upon an increased capacity of the mononuclear phagocytes of the immunized animal to destroy or inhibit the growth of tubercle bacilli The present study makes this conclusion more nearly certain For, in the former studies it was found that the fate of the bacilli of primary infection or of reinfection is greatly dependent upon the organ in which they are focalized Thus, when immunity develops in the course of a first infection, which had invaded all the organs simultaneously *via* the blood stream, the bacilli may be effectively destroyed in the liver, spleen, and bone marrow but not in the lung and kidney It is conceivable that forces, apart from the inherent properties of the phagocytic cells that harbor the bacilli, resulting from the structures or functions of the organs and acting as environmental factors affect the behaviour of these cells and account for the observations In the experiments here recorded it has been clearly demonstrated that immunity in tuberculosis is associated with an increased bacteriostatic property acquired by the mesenchyme cells in general that is independent of the organ environment in which the cells are situated, for it is also present outside of their influence

To what extent immune body fluids can impart to normal cells increased bacteriostatic effects is not entirely answered by these experiments for there was no continuous renewal of immune body fluids in the medium of growth of the normal cells that were originally introduced into the chambers with immune serum One especially hesitates to accept the inconstant results of this experiment in view of the fact that it has been clearly demonstrated that when bacilli situated extracellularly are continuously exposed to the body fluids of immune animals *in vivo* (2, 4) they are definitely inhibited in their growth by comparison to bacilli similarly exposed to normal body fluids.

CONCLUSIONS

1 Mononuclear phagocytes of immunized animals that had ingested tubercle bacilli *in vivo* and had subsequently been transplanted and grown in the environment of a normal animal continue to inhibit the multiplication of the microorganism in their cytoplasm in the absence of immune body fluids

2 Mononuclear phagocytes of immunized animals that had ingested tubercle bacilli *in vitro* in the presence of immune serum inhibit the multiplication of the microorganism in their cytoplasm to a much greater extent than cells of

normal animals that had ingested the bacteria in the same medium and had grown in a similar environment

3 The presence of immune serum during the *in vitro* ingestion of tubercle bacilli by mononuclear phagocytes of normal animals does not regularly endow them with increased bacteriostatic properties for the microorganism Whether or not continued sojourn of normal cells in immune body fluids will confer upon them such properties has not been determined

4 Mononuclear phagocytes of immunized animals that had ingested tubercle bacilli *in vitro* in a medium of normal serum and had subsequently grown in an environment devoid of immune body fluids inhibit the multiplication of the microorganism in their cytoplasm to a much greater extent than do normal cells under the same conditions

5 Active tuberculosis confers on the mononuclear phagocytes themselves increased bacteriostatic properties for the tubercle bacillus which are independent of the immune body fluids or of the organ environment in which they grow [1]

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[1] Preliminary reports of the present work were presented before the Eastern Pennsylvania Chapter of the American Society of Bacteriologists on Nov 28, 1939 (*J Bact* , 1940, 39, 339) and before the American Association of Pathologists and Bacteriologists, April 10, 1941 (*Am J Path* , 1941, 17, 636) Since its completion Kallós (18) has published qualitative tissue culture studies confirming these quantitative results

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EXPLANATION OF PLATES

All microphotographs were prepared from tissues stained by the Ziehl-Neelsen method and counterstained with hematoxylin The magnifications are about × 1400

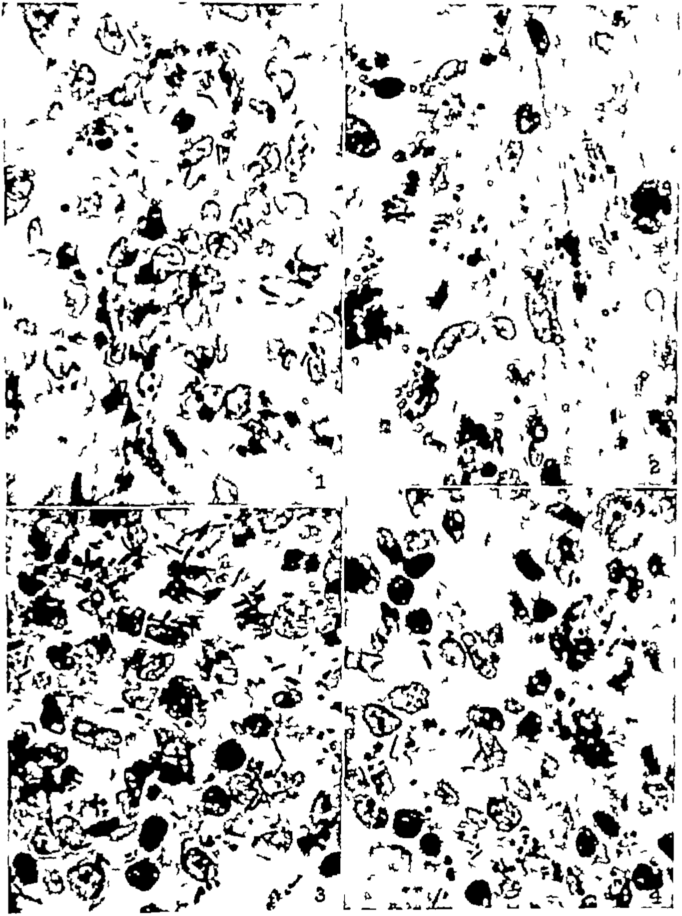
PLATE 5

FIG 1 Bone marrow of normal rabbit, the reticular cells of which had ingested tubercle bacilli and carbon particles *in vivo*, growing in the anterior chamber of the eye of a normal rabbit (Experiment 7, Table I*b*) 59,800 colonies were cultured from 100 mg of this tissue at the time of implantation After 14 days incubation, 66,000 colonies were cultured from 100 mg of implanted iris Ratio between the number of colonies in iris and that in the original inoculum, 1 1 Rapidly and diffusely growing mononuclears containing carbon particles and numerous, long, well stained tubercle bacilli in their cytoplasm

FIG 2 Bone marrow of immunized rabbit, the reticular cells of which had ingested tubercle bacilli and carbon particles *in vivo*, growing in the opposite anterior chamber of the same host that harbored the normal cells shown in Fig 1 91,800 colonies were cultured from 100 mg of this tissue at the time of implantation After 14 days incubation, 16,100 colonies were cultured from 100 mg of implanted iris Ratio between the number of colonies in iris and that in original inoculum, 0 17 The reticular cells of the surviving marrow contain carbon particles, one such cell, at the left margin of the photograph below its center, contains a mass of such particles The original fat spaces of the marrow are retained and may be seen in the lower left and both upper corners of the microphotograph Tubercle bacilli are very rare Two microorganisms, poorly stained and short, are seen lying at an obtuse angle to each other near the right margin of the photograph, at about its center

FIG 3 Phagocytic mononuclears of a normal animal, that had ingested tubercle bacilli and carbon particles *in vitro* in the presence of normal serum, growing in the anterior chamber of a normal host (Experiment 1, Table II*b*) 233,000 colonies were cultured from 100 mg of cells at the time of implantation After 14 days incubation, 240,000 were cultured from 100 mg of implanted iris Ratio between the number of colonies in iris and in the original inoculum, 1 03 Diffusely growing, carbon-containing, mononuclears swarming with long, deeply stained, tubercle bacilli

FIG 4 Phagocytic mononuclears from the same normal animal, as those shown in Fig 3 that had ingested tubercle bacilli and carbon particles *in vitro* in the presence of immune serum growing in the opposite anterior chamber of the same normal host that harbored the cells shown in Fig 3 380,000 colonies were cultured from 100 mg of cells at the time of implantation After 14 days incubation, 113,000 colonies were cultured from 100 mg of implanted iris Ratio between the number of colonies in iris and in the original inoculum, 0 30 Diffusely growing, carbon-containing mononuclears with fewer and less well stained tubercle bacilli in their cytoplasm than those shown in Fig 3



(Lurie Mechanism of immunity in tuberculosis)

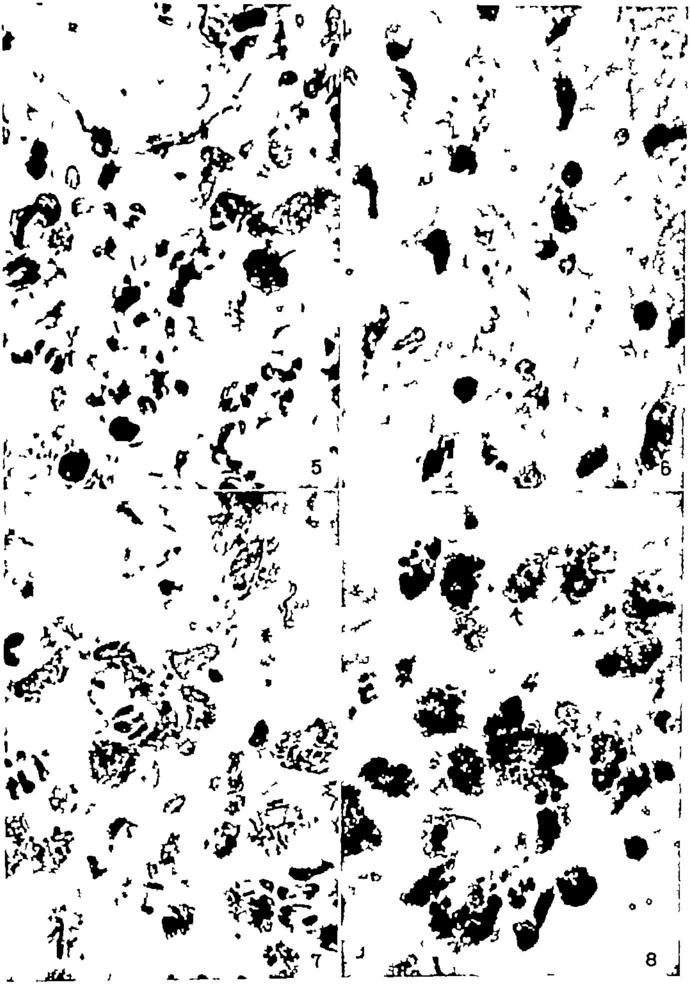
PLATE 6

FIG 5 Phagocytic mononuclears from a normal animal that had ingested tubercle bacilli and carbon particles *in vitro* in the presence of normal serum, growing in the anterior chamber of a normal host (Experiment 2, Table III *b*) 56,000 colonies were cultured from 100 mg of cells at the time of implantation After 14 days incubation, 7,300 colonies were cultured from 100 mg of implanted iris Ratio between the number of colonies in iris and in original inoculum, 0 13 Large mononuclears with large vacuoles in their carbon-containing cytoplasm, infiltrated with necrotic polymorphonuclear leukocytes The bacilli are numerous Two large skeins of bacilli, one of which is U shaped, can be seen in the mononuclears near the left margin of the microphotograph at about its center

FIG 6 Phagocytic mononuclears from an immunized animal, that had ingested tubercle bacilli and carbon particles in the presence of normal serum, growing in the opposite anterior chamber of the same normal host that harbored the cells shown in Fig 5 65,000 colonies were cultured from 100 mg of cells at the time of implantation After 14 days incubation, 860 colonies were cultured from 100 mg of implanted iris Ratio between the number of colonies in iris and in original inoculum, 0 013 There are large mononuclears with very small round vacuoles, some containing carbon particles with a structure characteristic of epithelioid cells Such a cell, with a peripheral dark staining and a central light staining cytoplasmic zone, is seen near the left of the photograph at about its center In the cytoplasm of a cell below the former one can see a very short, diploid tubercle bacillus, the only one that could be found in this field There are no infiltrating polymorphonuclears

FIG 7 Phagocytic mononuclears from a normal animal, that had phagocyted tubercle bacilli and carbon particles *in vitro* in the presence of immune serum, growing in the anterior chamber of a normal host (Experiment 6, Table IV *b*) 93,000 colonies were cultured from 100 mg of cells at the time of implantation After 14 days incubation, 810,000 colonies were cultured from 100 mg of implanted iris Ratio between the number of colonies in iris and in original inoculum, 8 71 Mononuclears, infiltrated with and phagocyting polymorphonuclears containing numerous tubercle bacilli and dispersed carbon particles in their cytoplasm are seen

FIG 8 Phagocytic mononuclears from an immunized animal, that had ingested tubercle bacilli and carbon particles *in vitro* in the presence of the same immune serum in which were suspended the normal cells shown in Fig 7, growing in the opposite anterior chamber of the same normal host that harbored the normal cells also shown in Fig 7 93,000 colonies were cultured from 100 mg of cells at the time of implantation After 14 days incubation, 5,300 colonies were cultured from 100 mg of implanted iris Ratio between the number of colonies in iris and in original inoculum, 0 057 Large mononuclears, heavily laden with masses of agglutinated carbon particles in their cytoplasm may be seen Tubercle bacilli are very difficult to find One very short, comma shaped bacillus, indicated by the arrow, can be seen in the cytoplasm of the cell near the upper right corner of the photograph just below the oval mass of carbon particles



(Lurie Mechanism of immunity in tuberculosis)

SEROLOGICAL REACTIVITY OF HYDROLYTIC PRODUCTS FROM SILK

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It has been briefly reported that precipitin reactions of antisera produced with heteroproteose are inhibited by peptic proteoses, small enough in molecular size to pass collodion membranes (1, 2) The purpose of these investigations was to get information on the smallest portions of a protein molecule that still are capable of specifically combining with antibodies and, finally on the chemical structure of these determinant groups, questions as yet undecided In resuming this line of work split products of silk, chosen as an example of a fibrous protein with a comparatively simple amino acid make-up, were examined and again inhibition reactions were observed with substances resulting from enzymatic digestion of this protein (see Bergmann and Niemann, 3) It was further seen that such reactions are obtained also with products of acid hydrolysis [1]

The preparation of antisera for silk presented some difficulty at first but on injection of silk, dissolved with the aid of acid and after neutralization adsorbed onto charcoal immune sera were obtained (4*a*) An attempt with the method briefly mentioned by Fell (4*b*) was unsuccessful

Methods and Materials

Immunization—6 gm of degummed silk were dissolved by stirring for about 4 minutes in 60 cc concentrated hydrochloric acid and the solution diluted with 10 volumes of water The solution was neutralized to weak acid reaction to Congo red with 10 per cent NaOH added in portions to avoid too high a temperature The solid material which separates was allowed to settle and the supernatant fluid syphoned off The sediment was washed by suspending in a large volume of water and after settling was centrifuged for a short time—at very low speed to prevent the formation of clumps that subsequently would be difficult to bring into solution. The precipitate was then taken up in 45 cc. of water and dissolved by adding dropwise dilute NaOH while stirring avoiding an excess The solution was brought back with dilute HCl to weakly alkaline litmus reaction [2] This solution was used for the sero-

[1] This observation was made in our laboratory by Dr R. F Clutton

[2] An electrophoretic experiment was kindly carried out by Dr L G Longsworth The opalescence of the solution interfered with the observation but the material seemed to be homogeneous and had a mobility of $-1\ 8 \times 10^{-5}$ cm /sec./volt/cm. in 1 per cent solution in phosphate buffer of 0 1 ionic strength and pH 8 0

logical tests and for immunization after adsorption to blood charcoal The charcoal was washed several times with water, dried, sterilized, suspended in saline solution, and thoroughly mixed with the silk solution, using 45 gm of charcoal for the solution made from 12 gm of silk (for preservation 0 5 per cent phenol was added) Immunization was carried out by repeated intraperitoneal injection of rabbits, each receiving at 5 day intervals 15 cc of a 6 fold dilution of the suspension in saline, a dose containing about 25 mg of silk and 0 35 gm charcoal Sera of moderate strength were obtained after 6 to 12 injections

Hydrolysis and Fractionation [3]—200 gm portions of degummed silk were dissolved, with stirring, in 1 liter of 50 per cent (by volume) sulfuric acid, and the solution was allowed to stand at 26–30°C for 4 hours 2 liters of water were added, the solution (for convenience) kept overnight in the ice box and then dialyzed in cellophane tubing against running water for 3 hours The remaining sulfuric acid was then removed with barium carbonate, in later runs with barium hydroxide, and any excess of barium was subsequently removed with sulfuric acid The barium sulfate was filtered off on Buchner funnels and washed with boiling water The combined filtrates and washings were evaporated in small portions on the steam bath to a small volume, the reaction during evaporation being kept slightly acid to litmus The syrupy solution was placed in cellophane tubes and dialyzed in the ice box against 2 volumes distilled water with changes of water at the intervals stated The outer fluids were evaporated in small portions on the steam bath, and finally were precipitated with acetone, filtered, and dried The first three diffusates, after dialysis for 3, then 5, then 7 days, were combined Yield 1200 gm from 4800 gm silk used (The materials obtained on further dialysis are not considered in the following, also, in the description of the fractionation procedures only those substances are taken account of that were selected for use in the serological tests presented)

The substance was boiled up with 2400 cc of water, cooled to room temperature, and a little toluene added as a preservative After standing overnight at room temperature an insoluble portion was removed from the solution (A) This sediment was again extracted with water as above (solution B) To solution A 1½ volumes of alcohol were added and the mixture kept in the ice box for 48 hours The resulting precipitate was filtered in the cold, washed with acetone, and dried [4] From the filtrate a 2nd fraction was obtained by addition of $^1/_5$ volume of alcohol (A_1, 102 gm) The supernatant was evaporated to a small volume and precipitated with acetone (A_2, 350 gm) Solution B was precipitated with 2 volumes of alcohol (precipitate B_1, 35 gm) A second precipitate was obtained by adding 3 volumes of alcohol and the supernatant fluid was evaporated to a small volume and precipitated with acetone (B_2, 100 gm) A_1 was further fractionated by successive addition of 1, ½, and

[3] In studies on the production of silk hydrolysis several dipeptides and a tripeptide (alanylglycyltyrosine) have been isolated in pure state (*cf* 3) A number of other split products have been described (5, 6)

[4] The same procedures dissolving in 2 volumes of water except when stated otherwise, precipitation, drying, were followed in the other fractionations The volumes of alcohol used in the fractionation are given in terms of the volume of the original aqueous solutions

1 volume of alcohol, and the last precipitate (11 gm.) was similarly refractionated with $\frac{1}{2}$, $\frac{1}{2}$ and $\frac{3}{4}$ *volume of alcohol.* The *3rd fraction* is designated as preparation I.

A_2 and B_2 were joined, dissolved, and the solution precipitated with 3 volumes alcohol, the precipitate (86 gm.) was dissolved in 3 volumes of water and again precipitated with $1\frac{1}{2}$ volumes of alcohol. This substance (35 gm.) dissolved in 4 volumes of water, was separated into fractions which precipitated with $\frac{3}{4}$, 1, 2 and 3 volumes alcohol. The last of these fractions, preparation II, amounted to 1.5 gm.

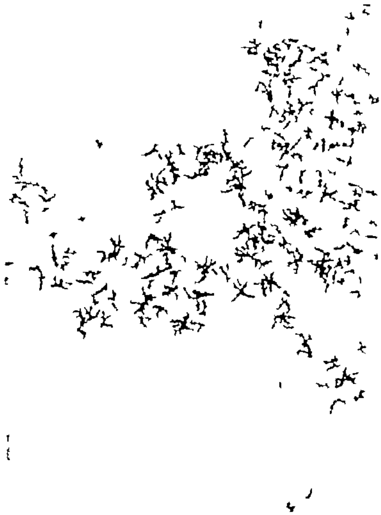

FIG. 1. Substance II from hydrolysis products of silk fibroin. × 240.

The precipitate B_1 was redissolved in 6 volumes of water and precipitated with an equal volume of alcohol (preparation III, 7 gm.).

A number of serologically active preparations were analyzed for total nitrogen and amino nitrogen in order to obtain estimates of the molecular size. The nitrogen analyses were made by the gasometric Kjeldahl method according to Van Slyke (with a modification devised by Goodner[5]). The amino nitrogen determinations were made by the Sörensen method using glass electrode measurements instead of indicators.

[5] Personal communication.

Glycine was determined with nitranilic acid (7), tyrosine by Bernhart's technique (8), and serine and alanine by the method of Fromageot and Heitz as modified by Desnuelle (9) (Deamination followed by oxidation and colorimetric determination of acetaldehyde)

Substances Tested—Substance I had microscopically a crystalline appearance (similar to that shown in Fig 1) when separating in the cold from solution in dilute alcohol It contained 17 34 per cent N, 2 47 per cent NH_2-N, which would correspond to peptides with seven amino acids and with molecular weights of about 600 The ash was 1 9 per cent, containing a considerable proportion of Ca and SO_4 To obtain an indication of the degree of homogeneity, the material was dialyzed in a cellophane bag against small volumes of water, the outer fluid being removed, and replaced after 18, 47, 90, and 138 hours The ratio of total N to amino N was 9 in the final inner fluid which contained only 4 per cent of the starting substance and varied from 7 3 in the first to 8 1 in the last diffusate That the molecular weight values of the fractions (about 580 to 640) calculated from the amino N are of the correct order of magnitude was confirmed by determination of the freezing point depression (made with the second diffusate) which gave even a lower value, but since complete analytical data are lacking an accurate correction for salt (ash 2 9 per cent) was not possible

Substance II had likewise a crystalline appearance, as shown in Fig 1 However, an x-ray picture failed to give definite evidence for crystalline structure [6] On analysis 17 76 per cent N, 2 26 per cent amino N, (and 2 57 per cent ash) were found indicating octapeptides Analyses for amino acids gave 48 8 per cent glycine, 35 6 per cent alanine, and 1 2 to 1 3 per cent tyrosine, the latter probably ascribable to some peptide present in small quantity

Substance III separated from the solution in the form of shiny balls as seen microscopically Analysis showed 18 11 per cent N, 1 53 per cent amino N, and 0 79 per cent ash, pointing to peptides with a chain length of 12 amino acids

FINDINGS

The preparations just described were tested serologically by inhibition reactions (Table I)

It is seen that all three substances inhibited the precipitation of silk antigen by the antisera though to somewhat different degrees (Still other fractions gave positive reactions likewise but were not further studied) The strongest inhibition was obtained with substance III which from the analytical results appeared to have the largest molecular size The specificity of the reaction was shown by tests, given in Table II, with several heterologous antiprotein sera and their corresponding antigens in which also a higher concentration of the substances was used In order to determine whether the activity of

[6] Strong reflections were observed corresponding to 4 67 and 4 15 Å u and a weak reflection at about 3 7, but they may be due to the ash content For this examination I am indebted to Dr I Fankuchen

TABLE I

To 0.2 cc. of a 1 10 000 silk solution were added 0.05 cc. of solutions of the substances tested for inhibition and then 2 drops of immune serum

Readings were taken after 15 minutes at room temperature (1st line) and after 1 hour (2nd line)

Solution tested for inhibition	Substance I	Substance II	Substance III	Control
per cent				
1	0	0	0	$\underline{+}$
	0	0	0	+
1/2	f tr	f tr	0	
	tr	$\underline{\text{tr}}$	f tr	
1/4	$\underline{\text{tr}}$	$\underline{\text{tr}}$	f tr	
	±	$\underline{+}$	f tr	
1/8	(±)	(±)	$\underline{\text{tr}}$	
	+	+	$\underline{+}$	

The intensity of the reactions is indicated as follows 0 f tr (faint trace) tr (trace) $\underline{\text{tr}}$ (strong trace) ±, $\underline{+}$ + +±

TABLE II

Test as in Table I using 1 drop of the suitably diluted immune sera and the respective homologous antigens in a concentration of 1/10 000

Readings were taken after 1 hour at room temperature.

Immune sera against	Substance II		Substance III		Control
	1 per cent	2 per cent	1 per cent	2 per cent	
Chicken ovalbumin No I diluted 1 3	±	±	±	±	$\underline{+}$
No II diluted 1 2	+±	+±	+±	+±	+±
Horse globulin No I diluted 1 3	$\underline{+}$	$\underline{+}$	$\underline{+}$	$\underline{+}$	+
No II undiluted	+	+	+	+	+

preparation III might be due to a substance of high molecular weight, a diffusion experiment was carried out in the following manner

6 gm of the substance were dissolved in 10 volumes of water by heating, cooled to room temperature, and some undissolved material (0 315 gm) filtered off The solution was placed in a cellophane bag and dialyzed in the ice box against 1⅓ volumes of water which was changed at the intervals stated The diffusates were evaporated to a small volume and precipitated with acetone

	Taken after	Weight	Total N	Amino N	$\frac{N}{\text{Amino N}}$
	hrs	*gm*	*per cent*	*per cent*	*per cent*
1st diffusate	18	2 2	17 54	1 67	10 5
2nd "	24	1 44	17 78	1 56	11 4
3rd "	30	0 82	17 60	1 47	12 0
4th "	43	0 45	17 51	1 51	11 6
5th "	74	0 27	17 19	1 30	13 2
Residue in bag		0 25	16 76	1 08	15 5

Fractions 2 and 3 were analyzed for individual amino acid and gave the values shown (R = ratio of nitrogen of each amino acid to NH_2 nitrogen in the original substance, indicating the number of molecules of the amino acid)

	2nd diffusate		3rd diffusate	
	per cent	*R*	*per cent*	*R*
Glycine	49 7	6 0	46 8	5 9
Alanine	26 4	2 7	25 3	2 7
Serine*	21 2	1 8	22 4	2 0
Tyrosine	18 6	0 9	18 1	1 0

* This value would include other hydroxyamino acids (threonine) (v 10)

Although the substance III is certainly not homogeneous, it would appear that the fractions 2 and 3, which are similar in composition, consist largely of peptides with a chain length of twelve amino acids, and this receives support from the amino acid composition which, incidentally, is not far from the proportions found in silk fibroin[7] (Bergmann and coworkers, (11, 12))

The results of inhibition tests with the fractions obtained by diffusion are given in Table III and show that there was no definite difference in the reactions of the five diffusates, the residue gave a somewhat stronger inhibition On the whole the experiment renders it very improbable that a small quantity of a substance with high molecular weight was responsible for the specific inhibitions observed Thus the analytical result showed that some separation with respect to molecular size had been obtained, as indicated by the figures for

[7] A non-crystalline substance, probably a tetrapeptide isolated by Fischer and Abderhalden (5) contained two glycine, one alanine, and one tyrosine residues

the 1st and 5th diffusates, while this difference is not reflected in the inhibition effects

TABLE III

Inhibition tests with diffusates of substance III.
Tests as in Table I.

Diffusates	1	2	3	4	5	6	Control
per cent							
1/2	0	0	0	0	0	0	+
	f tr	f tr	f tr	f tr	f tr	0	+
1/4	tr	tr	tr	tr	tr	f tr	
	tr	tr	tr	tr	tr	f tr	
1/8	±	±	±	tr	±	tr	
	+	+	+	+	+	+	

Continuation of the work in the direction of obtaining more homogeneous preparations of silk peptides is desirable as well as further investigations on other proteins

The author is indebted to Mr B Meier for his assistance

SUMMARY AND CONCLUSIONS

The foregoing experiments show that products of the hydrolysis of silk that consist of peptides having, from amino nitrogen determinations, molecular weights from about 600 to 1000 were capable of inhibiting the reactions of pre cipitin sera for silk. From the results it may reasonably be inferred that silk fibroin contains determinant structures not larger than the peptides examined, which probably consist of not more than 8 to 12 amino acids That similar relations may obtain with other proteins is not improbable in view of results previously reported with dialyzable split products

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SYNTHESIS OF INOSITOL IN MICE

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Since it has been shown (1, 2) that inositol possesses vitamin activity, it has been desirable to investigate the metabolism of this compound Some studies have been made previously, but the work has been seriously limited by the lack of suitable methods of quantitative estimation With the development of a satisfactory micromethod for the estimation of inositol (3), it has been possible to study the metabolism of this substance in some detail In particular, it has been possible to show that while mice require inositol in the diet, they are able to synthesize the compound when none is ingested Furthermore, the site of synthesis has been indicated, and a possible explanation for the numerous spontaneous cures of alopecia (4) has been discovered Finally, the influence of pantothenic acid on the metabolism of inositol has been demonstrated

EXPERIMENTAL

Method of Analysis —Inositol determinations were made according to the method recently described by Woolley (3) Individual mice were killed with chloroform, weighed and suspended in 50 cc. of HCl of such concentration that the final suspension was 18 per cent HCl The mixture was refluxed for 6 hours and extracted twice with ether The analysis for inositol was then conducted on the aqueous phase as previously described. In every case in which animals had received a diet containing inositol they were fed a ration free of this substance for 3 days before analysis. This was done in order to remove ingested inositol from the intestinal tract.

Variation in the values observed for individual male weanling mice is illustrated by the data given in Table I

Synthesis of Inositol in Mice

In order to determine whether mice were able to synthesize inositol, the following experiments were performed

Forty five weanling male mice were fed a highly purified diet composed of sucrose inorganic salts casein, cod liver oil, corn oil thiamin, riboflavin vitamin B_6 nicotinic acid choline, and pantothenic acid. The composition of this diet has been described (2)

Ten mice were individually analyzed and the average inositol content of a weanling mouse was thus obtained (see Table II) After the animals had been

fed the purified ration for 2 weeks ten more animals were analyzed A similar group was analyzed at 4 weeks and another group of five at 6 weeks The inositol content of an average mouse at biweekly intervals on this diet is shown in Table II It can be seen that the mice increased in total content of inositol per mouse even though none of this substance was ingested For purposes of comparison the average inositol content of six mice raised on stock rations for 4 weeks after weaning is included in Table II The inositol content of six

TABLE I

Inositol Content of Individual Weanling Male Mice

Mouse No	Live weight	Inositol content
	gm	*gamma/mg*
240	7 5	0 50
241	8 5	0 50
242	8 5	0 47
240A	8 5	0 47
345	7 5	0 48
340	7 5	0 50
341	8 5	0 50
342	8 5	0 47

TABLE II

Inositol Content of Mice Fed Rations with and without Inositol

Description of mice	Average live weight	Inositol content	Total amount of inositol
	gm	*gamma/mg*	*mg*
Weanlings	7 0	0 49	3 4
2 wks without inositol	15 0	0 35	5 3
4 wks without inositol	24 0	0 36	8 6
6 wks without inositol	35 0	0 35	12 3
4 wks on stock ration	25 0	0 39	9 8
4 wks with inositol	23 0	0 54	12 4

animals fed the highly purified ration plus 100 mg of inositol per 100 gm of ration for 4 weeks after weaning is also shown

The experiment was repeated with a second group of nine mice These animals increased in inositol content on the average from 4 mg to 12 mg in 6 weeks The experiment was again repeated using eight animals These increased in total inositol content from 4 mg to 11 4 mg

In the presence of adequate pantothenic acid one characteristic of inositol deficiency was the variation in gain of weight which individual animals exhibited Another characteristic was that, at about the 6th week of the experimental period, many showed a precipitous loss of weight Unless inositol was fed when this loss of weight began, death resulted The rate of loss was

as great as the rate of gain had been before the decline began A mouse typical in this respect was No 313, which gained from 9 5 gm. to 29.5 gm in 5 weeks In the following 10 days the weight declined to 21.5 gm 100 mg of inositol per 100 gm of ration were added, and in the next 8 days the mouse increased in weight to 27 0 gm When inositol was not administered to similar animals, death resulted after their weight had decreased to 15 to 20 gm Death was averted only when inositol was fed soon after the loss of weight became apparent In several cases these phenomena were not accompanied by alopecia at any stage of the experiment, and in no case was loss of hair observed during the period of precipitous loss of weight. Thus inositol deficiency was not invariably accompanied by alopecia.

The low incidence of alopecia has been reported on previous occasions (4) Examination of the data for individual mice in the first experiment described above demonstrated again that inositol deficiency cannot be produced in all animals In contrast to the uniformity in content of inositol of weanling mice (Table I) it was found that several of the animals kept on the inositol free ration had a low content. Thus it was low in two out of the ten analyzed after 2 weeks (0 24 and 0 27 gamma per mg , as compared to 0.35 for the average of the group) and after 4 weeks in three out of ten (0.30 0 29, 0.31 gamma per mg) In an independent group of seven mice three showed precipitous loss of weight after 7 weeks in a group of five mice two behaved similarly, while in a third group of seven two lost weight as described.

Relation of Pantothenic Acid to the Synthesis of Inositol —In order to investigate more fully the relationship of pantothenic acid and inositol to alopecia (4) the following experiments were performed A group of twenty two weanling mice were fed the above ration, minus pantothenic acid Ten animals were analyzed at the beginning of the experiments The remaining twelve were analyzed 3 weeks later The average content of inositol in the first group was the same as that previously recorded in Table II for weanling mice (0 49 gamma per mg , 4 0 mg per mouse) The average values in the second group were 0 24 gamma per mg and 4.3 mg per mouse Thus the inositol content per unit weight decreased and the total amount of this substance in the mouse remained approximately the same

The experiment was repeated twice with groups of six mice with the same result as in the first trial In a fourth experiment with 12 mice the content of inositol did not decrease significantly (0 48 to 0 41 gamma per mg) in 2 weeks, undoubtedly because of the small gain in weight of the group (from 6.5 gm to 10 gm) That is, the quantity of inositol contained in a mouse at the start and presumably carried through the test period was not distributed in as much tissue as in the other experiments.

Addition of pantothenic acid to the diet of animals deficient in this vitamin resulted in the synthesis of inositol A typical experiment was as follows

A group of fifteen mice were fed the ration deficient in pantothenic acid for 2 weeks The average content of inositol at the beginning was 0 49 gamma per mg and the total amount in the average mouse was 4 mg After the mice had been deficient in pantothenic acid for 2 weeks the content of inositol had decreased, but the total amount in a mouse was the same as at the beginning (judged from the average of two mice analyzed) Pantothenic acid was then restored to the diet Two to 3 weeks after the restoration the typical alopecia of inositol deficiency made its appearance in seven of the mice Four out of the seven hairless individuals exhibited spontaneous cure of the alopecia 2 to 3 weeks after its appearance even though no inositol was fed Analyses of these spontaneously cured animals revealed that in them inositol had increased to normal values (0 39 gamma per mg , 11 7 mg per mouse) Analyses of those which remained hairless showed that they had not increased markedly in content of inositol

Microbial Synthesis of Inositol

In an effort to discover the reason for the spontaneous cures of alopecia which were observed (4, and above) the following experiments were performed

The intestinal tract of a mouse which had exhibited spontaneous cure of alopecia was removed aseptically and placed in 10 cc. of a synthetic medium This medium had the composition described by Woolley (5), except that inositol and thioglycollic acid were omitted It was thus a highly purified mixture of all available growth factors and of glucose, inorganic salts, and amino acids The tube was incubated for 24 hours at 37°, and then a drop of the suspension was introduced into a second 10 cc. of medium of the same composition After 24 hours incubation, 1 cc. of this passage of culture of organisms from the intestinal tract was added to 500 cc. of medium of the same composition Incubation at 37° was continued for 60 hours The cells were then collected by centrifugation, hydrolyzed, and analyzed for inositol. The metabolism solution was concentrated under reduced pressure and similarly analyzed

As is shown in Table III, the organisms obtained by passage of a culture from the intestinal tract synthesized inositol and at least 80 per cent of the amount formed was retained in the cells The experiment was repeated with the same mixed culture (which had been stored at 0°) for an incubation period of only 16 hours Approximately the same quantity of inositol was formed as previously Hence the incubation periods in subsequent tests were 16 hours in length

The intestinal tract of a mouse from the same group of animals which had lost its hair and had not exhibited spontaneous cure was treated similarly The bacteria from this mouse showed a much smaller content of inositol (Table III) The experiments were repeated on two mice from a second run, one of which had become hairless but had then exhibited a spontaneous cure and the other of which had remained hairless The culture of organisms from the first mouse synthesized 0 42 gamma of inositol per cc of culture and that from the second mouse formed 0 12 gamma A third experiment with two more mice, cured and hairless like the others, was performed in the same manner and it was found that the values were 0 40 and 0 0 gamma per cc for the

culture from the spontaneously cured mouse and the hairless mouse respectively In this case the hairless mouse was examined as soon as alopecia developed, while in the other instances animals were taken which had been hairless for about one week

It has been found that the synthesis by the cells of the culture was not influenced by gramicidin and that a prominent intestinal inhabitant did not form inositol One of the cultures already tested, which was procured from a mouse which had exhibited spontaneous cure of alopecia, was inoculated into a medium of the composition described above, to which had been added 10 gamma per cc of crystalline gramicidin [1] A control test was done, using the same culture without the addition of gramicidin The cells from each medium were collected and analyzed for inositol As can be seen from Table III gramicidin did not

TABLE III

Inositol Content of Cultures of Intestinal Organisms

The numbers in parentheses indicate the length of the period of incubation

Material analyzed	Inositol content of culture
	gamma/cc
Cells of culture from spontaneously cured mouse (60 hrs.)	0 40
Supernatant fluid after centrifugation of the above cells	Less than 0 1
Cells of mixed culture from above mouse (16 hrs.)	0 38
Cells from the above culture grown in gramicidin	0 38
Cells of mixed culture from hairless mouse	0 18
Cells of *E. coli*	Less than 0 1

influence the synthesis The cells of one of the most important bacterial forms in the intestinal tract of the mouse, *Escherichia coli*, as grown in the purified medium, were analyzed and found not to contain inositol in demonstrable amounts (Table III)

Influence of Pantothenic Acid on Inositol Metabolism

The occurrence of alopecia in a high percentage of the mice to which pantothenic acid had been restored after 2 or 3 weeks of deficiency has been mentioned above It seemed likely that if inositol were fed from the beginning, this delayed appearance of alopecia might be prevented A group of ten mice were fed the basal ration referred to throughout this paper, from which pantothenic acid was omitted and which was supplemented with 100 mg of inositol per 100 gm After 2 weeks pantothenic acid was restored and inositol was omitted Two to 2½ weeks after this change typical alopecia developed in all but three of the animals. Analysis of three of the hairless mice showed that they contained little more inositol than did weanling mice (6 4 mg compared to 4.5 mg)

[1] We wish to thank Dr R. J Dubos for gifts of gramicidin.

Similarly, it had been found that two of the animals at the time that pantothenic acid was added were deficient in inositol even though they had received it in the diet (4 0 mg compared to 4 4 mg)

The experiment was repeated with the following modifications A group of six mice were fed the ration which contained inositol but no pantothenic acid After 2 weeks analysis of three mice again showed that they were deficient in inositol The other three mice were continued on inositol for 3 days following the addition of pantothenic acid Analyses of these revealed a normal content of the vitamin (0 42 gamma per mg) Thus it appeared that when pantothenic acid was absent from the ration inositol deficiency developed even though this substance was ingested Addition of pantothenic acid to the diet restored the inositol content of the mice to normal within 3 days

TABLE IV

Distribution of Free and Total Inositol in Various Natural Products

Except in the case of the extracts analyses were based on weights of undried samples

Material analyzed	Free inositol	Total inositol
	gamma/mg	*gamma/mg*
Normal mice (6 individuals)	0 25	0 41
Inositol-deficient mice (3 individuals)	0 20	0 30
Beef skeletal muscle	0 55	0 88
Beef brain	3 0	6 0
Beef pancreas extract	1 2	5 0
Aqueous alcohol extract of rice bran	0 37	0 53
Dialyzed rice bran extract	0 0	0 16

Free and Combined Inositol

Since it has been shown (6) that yeast is unable to respond to inositol esters, it was thought possible that the method for the estimation might be refined to differentiate free from combined inositol It has been shown (2) that liver contains a non-dialyzable, water-soluble substance which liberates inositol when treated with acid or alkali It has been found that yeast does not respond to this combination This has made possible the analysis of natural products for free inositol, separate from total inositol The difference between free and total represents combined inositol

For the analysis of mice for free inositol the following procedure was used

A mouse was ground and suspended in water An aliquot of this suspension was hydrolyzed with HCl and total inositol was determined in the hydrolysate as previously described A second aliquot of the suspension was heated in an autoclave (15 pounds for 15 minutes), centrifuged, and the precipitate washed with water Direct analysis of this liquid without acid hydrolysis gave a value for free inositol Since the combined inositol present in liver extract, brain extract, pancreas extract, and rice

bran extract was not rendered available to yeast by autoclaving, this procedure was used to coagulate proteins and destroy enzymes.

By this technique approximately 60 per cent of the inositol content of normal mice was found to be free Similarly, 50 per cent of the total inositol content of brain and 60 per cent of that in skeletal muscle was found to be free The proportion of free inositol in normal mice did not differ significantly from that in deficient animals. Some representative data are shown in Table IV In the case of tissues the value for free inositol represents the amount which can be extracted and does not include any free inositol which may be retained in insoluble residues

DISCUSSION

It can be clearly seen from the data in Table II that mice synthesized inositol when fed the purified ration. It appears that the presence of pantothenic acid in the ration was of importance for this synthesis Those animals which did not receive pantothenic acid failed to increase markedly in total inositol content even though they did gain in weight, whereas, when pantothenic acid was added to the ration, the inositol content of the mice increased It may be of interest to note that in no case of deficiency did the total amount of inositol in a mouse decrease even though the content per unit weight did fall Hence it was necessary that the animals should grow if the inositol deficiency was to be recognized by a decreased content of the vitamin per unit weight.

One site of inositol synthesis appears to be the intestinal tract When a mouse exhibited spontaneous cure of alopecia, a simultaneous increase in the total content of inositol took place Organisms cultivated from the intestinal tract of mice which had exhibited spontaneous cure synthesized inositol in the instances tested and to a much greater extent than did the organisms isolated by the same method of cultivation from hairless mice. Since the synthesis took place in the presence of gramicidin, which inhibits the growth of Gram positive bacteria (7) and since the gramicidin treated cultures on microscopic examination were found to be Gram negative, it is probable that the organisms responsible for the synthesis were Gram negative However, a prominent Gram negative organism of the intestinal tract, *E coli*, did not form inositol. Since inositol deficiency could be produced regularly in a small percentage of mice fed a deficient diet, it must be concluded that the inositol synthesized by such organisms was not sufficient to meet all of the requirements of the mouse, or else that, under the conditions studied, the organisms did not become established in all animals This latter hypothesis may explain more adequately why only some animals on an inositol free diet develop alopecia The data show further that the metabolism of inositol is influenced by pantothenic acid Mice did not increase in total content of inositol even when this sub-

stance was present in the diet unless pantothenic acid was also present It has not been established whether this phenomenon was due to failure of absorption or to some more obscure metabolic disturbance

SUMMARY

It has been shown that mice are able to synthesize inositol This synthesis was not observed when pantothenic acid was absent from the diet Cultures from the intestinal tract of animals which exhibited spontaneous cure of alopecia yielded microorganisms which synthesized much more inositol than did organisms isolated in the same fashion from the tracts of mice that had become hairless Some observations on the distribution of free and combined inositol have been made and it has been shown that several biological materials contain combined inositol It has been found that deficiency of inositol can develop even when inositol is present in the diet if pantothenic acid is omitted

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Hemolytic "units" were measured as in (3) both in the original sera and in the supernatants.

In order to supply the missing components for the fortification of human complement in the hemolytic titration fresh supernatants from the above C′ series were used, or, if these were not available, supernatants were quickly prepared as follows. For each milliliter of guinea pig serum specific precipitate was formed with about 0.02 mg. of egg albumin nitrogen and 0.1 to 0.2 mg. of rabbit anti-egg albumin nitrogen, or with similar amounts of Type II or Type III pneumococcus specific polysaccharide and homologous rabbit antibody. These quantities of specific precipitate have been shown to remove C′1, or the combining component(s), from complement. The mixture was centrifuged after the precipitate flocked and the supernatant was used as indicated.

TABLE I

Human Complement Titrations with and without Reinforcement

Human serum and dilution used	Guinea pig serum supernatant	Hemolysis by quantity of human serum dilution used:							
		0.05 ml.	0.075 ml.	0.1 ml.	0.15 ml.	0.2 ml.	0.25 ml.	0.3 ml.	0.4 ml.
B_1 1:30	−						ac	c	
" "	+			+++±	c				
C_1 1:10	−					+++±	c		
1:30	+			+	+++	c			
E_1 1:10	−	++		+++±	c	c			
" 1:40	+			+++	ac	c			
2_a 1:25	−					+	+++	+++±	
" "	+	+++	ac	c					
13_a 1:10	−					+++		ac	
" 1:30	+	+++	ac	c					
17_a 1:10	−				ac	c	c		
" 1:30	+		ac	c	c				
28–32_a 1:40	−			0		ac			c
" 1:320	+			0		++			c

Experiments

For the typical titrations given in Table I 0.2 ml. of hemolytic system (equal volumes of 5 per cent sheep red cell suspension and a dilution of hemolysin containing 2 or 4 "units") was added to each tube, followed by 0.05 ml. of the guinea pig serum supernatant, made up to double the original serum volume. Varying volumes of diluted human serum were then added, followed by saline to a total of 0.5 or 0.6

ml. The usual controls were run, including one with 0 1 ml of guinea pig supernatant with saline and hemolytic system. This was negative in every instance For each serum the first line in the protocol gives the titration without supplement, while the second line indicates the result of addition of the missing component or components

Lot D—In an earlier experiment C′ and iC′ blanks with saline alone showed no less N than corresponding blanks with antigen and serum alone Saline blanks were therefore dispensed with As neutralization of guinea pig C′ and iC′ had been found unnecessary, this step was also omitted with the human sera and earlier protocols are not given pH of C′, 7 38, pH of iC′, (after 50 minutes at 56°C), 8 21 Diluted anti-Pn III rabbit serum pool, B 40, 60 used S III, 0 04 mg per ml C′2 "titer" determined 1 week later after storage in CO_2 snow, 70 units per ml , C′1 "titer" (with guinea pig serum supernatant), 120 units Tubes held at 19–21°C for 2 5 hours, overnight in ice box before centrifuging

No of tubes	1	1	1	1	3	3	3	3	3	3
C *ml*	6 0	6 0						2 0	4 0	6 0
iC *ml*			6 0	5 5		2 0	6 0			
Serum dilution *ml*	1 0		1 0		1 0	1 0	1 0	1 0	1 0	1 0
S III dilution *ml*		1 0		1 0	1 0	1 0	1 0	1 0	1 0	1 0
Saline *ml*	1	1	1	1	6	4		4	3	1
N precipitated *mg*	0 012	0 012	0 018	0 024*	0 444 0 448 0 446	0 470 0 476 0 482	0 492 0 500 0 492	0 556 0 568 0 554	0 624 0 590 0 610	0 616 0 612 0 634
Mean	0 012		0 021		0 446	0 476	0 495	0 559	0 608	0 621
Subtraction of blank						0 007‡	0 021	0 004‡	0 008‡	0 012
Specific N precipitated *mg*						0 469	0 474	0 555	0 600	0 609
Subtraction of iC′ value								0 469	0 472§	0 474
C′ N precipitated *mg*								0 09	0 13	0 14
C′ N precipitated per ml C′ taken								0 045	0 033	0 023

* Calculated to 6 0 ml.

‡ Aliquot portion of blank on 6 0 ml.

§ Mean of the two iC′ series values

Hemolytic units left in each 2, 4, 6 ml. C′ supernatant respectively, <<40, <80, <<160

Data obtained with another portion (D′) of the same lot kept frozen a week longer, and then analyzed, are given in Fig 1

Lot E—Anti-Ea rabbit serum pool *d*, diluted to 3 5 volumes, (0 4 mg anti-Ea N per ml.) and Ea, 0 04 mg N per ml were used as the immune system C′2 "titer" of lot E, 70 units, C′1 "titer" (with guinea pig serum supernatant), 230 units Tubes centrifuged after 3 hours at 20–21°C

Portions of lot E, active and inactivated as in the previous section, were first treated with anti-Pn horse specific precipitate before addition of the rabbit system, as had been done in the study of guinea pig complement (3) The anti-Pn VIII horse serum H644 used had been precipitated with S III in another connection (7)

No. of tubes	1	1	1	1	3	3	3	3	3	3
C' ml	3 0	3 0					1 5	2 5	4 0	6 0
iC ml.			3 0	3 0		3 0				
Serum dilution ml.	1 0		1 0		1 0	1 0	1 0	1 0	1 0	1 0
Ea dilution, ml.		0 5		0 5	1 0	1 0	1 0	1 0	1 0	1 0
Saline, ml.	1	0 5	1	0.5	3					1
N precipitated, mg	0	(Lost)	0 010	0	0.394 0 386 0 396	0 404 0 396 0 384	0 460 0 464 0 464	0 490 0 484 0 476	0 506 0 512 0 500	0 534 0 524 0 540
Mean	0		0 005		0 392	0.395	0 463	0 483	0 506	0 533
Subtraction of blank						0 005	0	0	0	0
Specific N precipitated, mg...						0 390	0 463	0 483	0 506	0 533
Subtraction of iC' value							0.390	0 390	0 390	0.390
C' N precipitated mg							0 07	0 09	0 12	0 14
C' N precipitated, per ml. C' taken							0 047	0 036	0 030	0 023

* Since 3 0 ml. iC' gave the same value as the salt control series it is assumed that amounts of iC' corresponding to the other volumes of C' used would also have given the same value.

Hemolytic units left in each 1.5, 2.5, 4 6 ml. supernatant, respectively <7, <9 <12 20

and had been diluted to an anti S VIII content of 0 65 mg N per ml. After the reaction components were mixed, the tubes were allowed to stand 1 hour at 21°C

C' ml.	5	5	30		
iC', ml.				30	
Saline ml.			0 5	0 5	30 5
Anti Pn VIII horse serum, ml	0 8		5 0	5 0	5 0
S VIII 0 17 mg. per ml., ml		0 2	1 0	1 0	1 0
N precipitated from aliquot, mg	0 016*	0 016*	0 538 0 538 0 536	0 548 0 550 0 554	0 518 0 524 0 520
Mean	0 016		0 537	0 551	0 521
Total N precipitated, mg			2 15	2 20	2 08
Subtraction of blank, mg			0 10	0 10‡	
Specific N precipitated mg			2 05	2 10	2 08

* Entire precipitate from blanks used for analysis.

‡ C' and iC' blanks in the preceding experiment with serum E were approximately the same Mean blank (first 2 columns) × 6 used.

and were centrifuged in the cold. All supernatants were recentrifuged and the precipitates were washed 3 times with 10 ml. of chilled saline, dissolved in alkali, and rinsed into 20 ml. volumetric flasks. Aliquot portions of 5 0 ml. were analyzed for N

The recentrifuged C', iC', and saline supernatants from the above reaction were set up with the same Ea and rabbit anti Ea dilutions used in the earlier experiment

on untreated human serum E 6 ml. of each supernatant represented 4 9 ml of the original C′, ıC′, or saline

No of tubes	1	1	1	1	3	3	3
C′ supernatant, *ml*	6 0	6 0					6 0
ıC′ " *ml*			6 0	6 0		6 0	
Saline " *ml*					6 0		
Anti Ea serum, *ml*	1 0		1 0		1 0	1 0	1 0
Ea dilution, *ml*		1 0		1 0	1 0	1 0	1 0
Saline, *ml*	1	1	1	1			
N precipitated, *mg*	0 024	0 008	Lost	0 014	{0 392, 0 386, 0 382	{0 410, 0 406, 0 396	{0 502, 0 496, 0 524
Mean	0 016		0 014		0 387	0 404	0 507
Subtraction of blank, *mg*						0 014	0 016
Specific N precipitated, *mg*						0 390	0 491
Subtraction of ıC′ value							0 390
C′ N precipitated, *mg*							0 10

Hemolytic units taken, per 4 9 ml original C′, 1125
" " in C′ horse serum blank, " " 930
" " " C′-S VIII " " " 1030
" " " C′-S VIII-anti-Pn VIII supernatant, 960
" " " final supernatant, <16

Because of the apparent solubility effect in other experiments (see also Fig 1) a run was made with 30 ml. of C′ with the rabbit system. It was thought that at this volume the inhibiting effect of C′ excess might be great enough to mask the addition of C′ N and give N figures like those with S VIII and horse antiserum in spite of fixation of the C′ present A portion of lot H of human serum (C′1 "titer," 200 units per ml) was used as no more of lot E was available Anti-Pn-III rabbit serum B 40, diluted to 9 volumes S III, 0 19 mg per ml. Tubes allowed to stand at room temperature for 3 hours, in ice box overnight Analyses run as in protocol 2, lot E

C′, *ml*	10	30 0	
Saline, *ml*	2		30
Anti-Pn III rabbit serum, *ml*		5 0	5 0
S III solution, *ml*		1 0	1 0
N precipitated from aliquot, *mg*	0 032	{0 750, 0 756, 0 754	{0 570, 0 574, 0 566
Mean	0 032	0 753	0 570
Total N precipitated, *mg*		3 01	2 28
Subtraction of blank, *mg*		0 10	
Specific N precipitated, *mg*		2 91	2 28

The difference between the C′ and saline series, 0 63 mg of N indicates that the apparent solubility effect does not account for the values found with S VIII and anti Pn VIII horse serum

Lot G′—A comparison was made of the C′ N removed from this serum by two different immune systems, Ea anti Ea, and S II-anti Pn II. The former was that used in the preceding instance Ea solution 0 045 mg N per ml Anti Pn II rabbit serum 580, diluted to 4 volumes with saline S II, 0 1 mg per ml. C′2 'titer" of lot G′, 80 units C′1 "titer" (with guinea pig serum supernatant), 800 units

No. of tubes.	1	1	1	3	3	3	2
C′, *ml.*	1 0	2 0	2 0			2 0	2 0
Anti Ea dilution, *ml*				1 0			1 0
Ea dilution, *ml*	0 2			1 0			1 0
Anti-Pn II dilution *ml*		0 5			1 0	1 0	
S II dilution, *ml*			0 5		1 0	1 0	
Saline, *ml*	0 2	0 5	0 5	2	2		
N precipitated, *mg*	0 006	0 006	0 002	0 448 0 438 Lost	0 120 0 146 0 150	0 198 0 198 0 210	0 528 0 526
Mean	0 012*	0 004		0 443	0 139	0 202	0 527
Subtraction of blank						0 004	0 012
Specific N precipitated, *mg*						0 198	0 515
Subtraction of saline blank value						0 139	0 443
C′ N precipitated, *mg*						0 06	0 07

* Calculated to 2.0 ml.

Hemolytic units left in each Ea system supernatant, <13 in Pn II system supernatant, 20

Lot 19₂—C′2 'titer' <75 units C′1 "titer, 400 units per ml. This human serum, containing antibodies to Type II pneumococcus, was preserved with 1 10 000 merthiolate and handled and analyzed so as to favor sterility Estimation of C′ N on 2.5 ml. samples with Ea-anti Ea as in previous examples gave 0 08 mg C′ removal was practically complete 4.5 ml. portions of the supernatants from the C′ analysis corresponding to 2.5 ml. of original serum, were each mixed with a solution of 0 06 mg of S II and allowed to stand in the ice box for 5 days since in this and other instances of the analysis of weak human sera precipitation increased visibly during the first few days Similar analyses were made with fresh serum 19₂ containing C Blanks were also run and deducted.

Specific N from 2 5 ml. containing C′,	0 117 mg
Anti-S II N from equivalent volume ' decomplemented serum	0 090 mg
Human C N removed by S II-human anti S II,	0 027 mg

C′1 units in supernatant calculated to original volume 160 per ml.

In this instance the human specific precipitate took up 30 per cent of its weight of C′ and removed more than one-half of the number of C′1 units present

DISCUSSION

It was shown in the introduction and in Table I that titration of human complement in the usual way does not yield the same information as with guinea pig complement and a new titration procedure for overcoming this difficulty was proposed It is evident from the Experimental part, however, that one need not remain content with relative figures, and that the quantitative estimation of complement combining component or components in absolute terms, or weight units (3) is applicable without modification to human sera

The amounts of complement nitrogen added to specific precipitates in fresh human serum are of the same order of magnitude as in guinea pig serum, but there seems to be little relation to the hemolytic titers of the various samples, whether these titers were obtained in the usual way or with added guinea pig serum supernatant The reason for this is not clear but may be related to the growing mass of evidence that other components of C′ as well as C′1 are taken up

When the present method for complement estimation (3) was proposed, it was made clear that the nitrogen determined was that of complement as defined by Muir (8) and that the designation C′1 N was a provisional one in accord with current belief that the combining component, or components, consisted mainly of "midpiece" or C′1 (9, 10) The inadequacy of this belief has often been maintained (11) It has now been shown in new work by Ecker and Pillemer (12) and by our own studies, which it is hoped soon to publish, that other complement components also add to specific precipitates The quantitative method therefore remains, as before, a valid one for the estimation of complement or, more strictly, complement combining components in weight units

Fig 1 shows that the apparent solubility effect is also present as in guinea pig complement If the lines in the figure are extrapolated roughly to zero volume they lead to the conclusion that the sera analyzed contain between 0 03 and 0 05 mg per ml of nitrogen due to complement combining component or components This might provisionally be taken as the normal range of fluctuation for human sera Many other samples, both from normals and from cardiac and hypertensive cases, have fallen within the same limits These are not shown in the chart, partly to avoid overcrowding, and partly because many of the analyses were carried out with a single volume of serum

It is also shown in the experiment on lot G′ that the quantity of C′ N found does not depend on the immune system used and is like that found in other sera with S III-anti-S III[3] The second portion of the experiment on lot E, however, shows that human complement does not add to a specific precipitate from anti-

[3] Irregular results with the S I-anti-Pn I rabbit serum system in guinea pig and human sera are under investigation

pneumococcus horse serum, and that in this respect, also, it resembles guinea pig complement (3, 13) The experiment shows, further, that the nitrogen ascribed to C′ cannot be due to insoluble or easily adsorbed material other than C′, since no more nitrogen was carried down by quite adequate amounts of the horse specific precipitate from C′ than from iC′ or saline Moreover the supernatant showed very nearly the same quantity of C′1 N, when subsequently treated with egg albumin rabbit anti-egg albumin as did the untreated portion of lot E

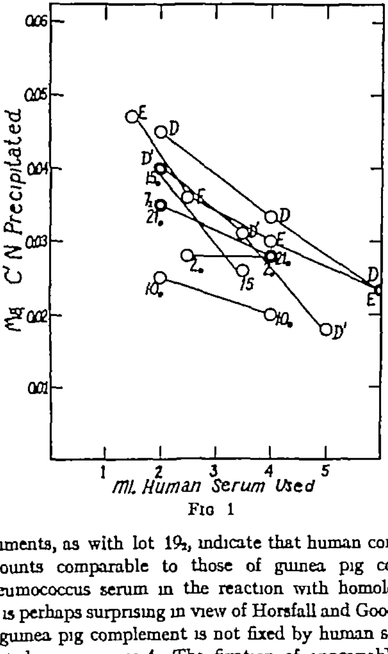

FIG 1

Initial experiments, as with lot 19₂, indicate that human complement may be fixed in amounts comparable to those of guinea pig complement by human antipneumococcus serum in the reaction with homologous polysaccharide This is perhaps surprising in view of Horsfall and Goodner's observation (14) that guinea pig complement is not fixed by human sera in reacting with encapsulated pneumococci.[4] The fixation of appreciable amounts of

[4] Fixation of human complement by human immune sera is evidently subject to unknown factors. Thus far 4 sera have shown precipitation of more specific N in the presence of human C′ than in its absence but in only 2 of these was the decrease in "titer" checked. Two other sera, on the other hand showed no difference in specific N and no decrease in "titer"

human C′ N in the S-human-anti-Pn system complicates the interpretation of these and other precipitin reactions in human sera It is quite likely that small quantities of antibody, such as might occur during convalescence or after active immunization, could easily appear to be doubled (*cf* 4) by the C′ N fixed during combination of the antibody with antigen and thus indicate too high an antibody content It is also possible that inactivation of human sera for a period long enough to abolish fixation of C′ N (50 minutes at 56°C) would damage small amounts of antibody Therefore it appears best, while these possibilities are being investigated on a large number of sera, to remove C′ by the method used in the experiments referred to, namely, by absorption with adequate amounts of an unrelated precipitating system, such as Ea-rabbit anti-Ea Attention is also called to the length of time (4 to 5 days at 0°C) required for complete separation of small quantities of specific precipitate from human sera, a period which seems to be lengthened, rather than diminished, by the coprecipitation of active complement As is stated in (3) flocculation even of relatively large amounts of specific precipitate from rabbit antibody is delayed by guinea pig C′ The same observation was made throughout the experiments with human C′ and in many instances the delay in flocculation was even more marked than with guinea pig C′

SUMMARY

1 A modified method is given for the titration of human complement so that C′1 titers are measured, as in guinea pig serum, instead of the C′2 titers yielded by the usual titration

2 The measurement of complement combining component or components in weight units, instead of relative terms, is carried out as in guinea pig serum and leads to similar values, 0 03 to 0 05 mg of C′ N per ml of human serum

3 Other similarities in human and guinea pig complements are noted and discussed

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THE EFFECT OF UNDERNOURISHMENT ON THE SUSCEPTIBILITY OF THE RABBIT TO INFECTION WITH VACCINIA*

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It is generally believed that the healthy, well nourished person is more resistant to disease than the individual who is already diseased or poorly nourished That such is not the case, however, has long been recognized in certain instances of viral infection Jenner (1) found that individuals with another disease were sometimes immune to vaccination Underwood (2) in 1799 pointed out that poliomyelitis attacked the finest children Rous (3) in 1911 reported that healthy fowls are more susceptible to infection with sarcoma virus than unhealthy ones Rivers (4) reviewing these and similar observations with viruses, has suggested that such behavior is not wholly unexpected and may be related to the extreme type of parasitism exhibited by viruses, namely, the dependence of viral disease on the multiplication of virus in susceptible cells.

At the present little is known concerning the factors upon which susceptibility of a cell to virus infection depends However, it is reasonable to suppose that a poorly nourished cell may be lacking in some of the materials necessary for the formation of new virus particles With this possibility in mind, it was decided to study quantitatively the effect of undernourishment on the susceptibility of the skin to infection with vaccinia In spite of the fact that the rabbit is an extremely unsatisfactory animal for metabolic experiments, it was chosen for this work because it is well suited for precise quantitative studies with vaccinia

The first experiments reported here confirmed fully the previously cited observations, in that they showed that simple undernourishment decreases the susceptibility of the rabbit's skin to infection with vaccinia However, it became apparent from other experiments that simple reduction of available nutrient materials is not the sole factor involved in this decreased susceptibility One other factor shown by the present experiments to be important is the amount of interstitial fluid present. In the presence of an increase of this

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fluid the virus particles tend to remain localized at the site of injection, this localization, as shown in an earlier paper (5), suggests that the virus comes into contact with fewer susceptible cells and the number of lesions is thereby reduced The opposite is true where the amount of interstitial fluid is either normal or reduced

The amount of interstitial fluid increases if animals are deprived of food but are permitted to drink water freely This increase is not the result of lowered plasma proteins, as these are unchanged It is prevented, however, if no water is administered by mouth or if physiological saline solution is administered parenterally It is therefore apparent that experimental techniques are at hand for studying the effects both of undernourishment and of variations in the amount of interstitial fluids This paper is a report of the influence of these factors on vaccinia infection

Methods and Materials

Animals—Normal white adult male rabbits weighing between 2 and 3 kilos were used.

Virus—The virus employed in the first 3 experiments was provided by Dr J W Beard This strain was derived from vaccine lymph originally obtained from the North Carolina Laboratory of Hygiene, Raleigh, and has been described in detail (6) This virus had been passed 50 times in rabbit skin It was purified as described by Craigie and Wishart (7) and Parker and Rivers (8) Measured amounts were rapidly lyophilized and sealed in a vacuum until used in these experiments

The virus used in the other experiments was a strain of vaccinia supplied by Dr T M Rivers This strain had been derived through serial passage through rabbit skin for a number of years and was well adapted to this animal This strain was purified as was the other strain It was not lyophilized but was kept frozen in dry ice until used

Conditions of Fasting—The rabbits to be fasted were kept in cages with wire mesh bottoms sufficiently large to prevent the eating of feces Three different regimes of fasting were observed in these experiments In the first, the rabbits received no food but were given free access to water In the second, neither food nor water was given For the third, the animals were fasted with access to water and in addition were injected intraperitoneally with 50 cc of physiological saline solution twice daily These regimes were maintained for 10 days before virus inoculation and for 48 hours afterward. The animals were then allowed such small amounts of food that their weights remained stationary The rabbits of the group deprived of both food and water were given free access to water as well as some food 48 hours after vaccination Estimations were made of plasma proteins in most of the fasted animals No significant changes were noted, so that this factor could be ruled out as an influence in the production of hydration

Seven days after virus inoculation all the rabbits were killed and necropsied. The animals listed in Table I as fasted were practically free of any subcutaneous, mesenteric, perirenal, or any other deposits of fat. Six of the 21 rabbits which were

fasted with water were found to have some fat and have been listed as incompletely fasted.

Controls—The control animals were kept on the usual stock diet. Normal deposits of fat were seen in all of them. Certain of the fasted rabbits which failed to lose fat served as additional controls A few of the rabbits developed diarrhea during the course of the experiment, some before and some after vaccination. These were excluded from the experiments since this complication had been seen to affect susceptibility to viral infection The incidence of this complication was as follows 3 of 21 control rabbits, 3 of 21 rabbits which were fasted but given water 3 of 9 rabbits which were fasted and given isotonic saline solution and none of the 9 rabbits which were given neither food nor water Two of this last group, however died of inanition

Virus Inoculation—Tests for susceptibility to infection were made by intradermal injection of 0 25 cc. of 7 fourfold dilutions of virus Each rabbit received 7 inoculations of each dilution or a total of 49 inoculations in each animal. The 50 per cent points were calculated by the accumulation positive and negative method of Muench and Reed (9)

Spread of Materials—This was studied by the intradermal injection of 0 5 cc. of an India ink suspension The spread of India ink was measured with a planimeter immediately after injection and at 1 2 and 4 hours afterward. Only the 4 hour readings are given. The technique of this procedure is given in detail elsewhere (10)

Throughout this paper the expression, fasted animals, will mean those which were deprived of food but given free access to water

EXPERIMENTAL

The Effect of Undernourishment—An experiment was done on the effect of the deprivation of food but not water on the susceptibility of the rabbit to viral infection Food was withheld from rabbits, but they were given access to water, and 3 were kept as controls. The results of inoculation of graded quantities of virus in this group are given under Experiment 1 in Table I In the 3rd column are the number of inoculations of each dilution injected and in the 4th the per cent change in weight occurring during fasting prior to inoculation Here it is seen that while the controls lost only 1 per cent of their weight the loss in the fasted animal was 19 per cent In the 5th column are given the 50 per cent points. The logarithm of the virus dilution corresponding to the 50 per cent point for the control animals is 6 85 (dilution 1 7,080 000) In contrast, that for the fasted animals was 5.88 (1 759,000) The difference in logarithms was 0 97, the antilogarithm of which is 9 3 Therefore, the fasted animals may be thought of as being more than 9 times as resistant to viral infection as the controls This result is well within the limits of the method of infectivity measurement and therefore indicates a definite increase in resistance related to fasting

Viewed solely in the light of response as measured by the respective 50 per cent points the data obtained in this experiment might well be construed as

indicative of increased resistance due solely to poor nutrition That this was not necessarily the case, however, was evident in associated findings For one thing, it was observed that the blebs raised by the injections of the virus preparations subsided much more slowly in the fasted animals than did those in the controls In earlier work (11) it was found that when blebs subside slowly the spread of particulate matter is correspondingly less than in instances where the blebs disappear rapidly This was of particular interest in the present problem for it has been observed (5) that localization of injected virus due to failure to spread is associated with diminished likelihood of infection A study was then made to learn whether or not the spread of India ink is altered in animals fasted as in the present experiment

Comparison of Susceptibility and India Ink Spread in Undernourished Rabbits—An experiment was carried out to find whether deprivation of food but not water actually localized the virus as the foregoing observations suggested This question is important for it has been shown (5) that localization of a virus diminishes the chance of infection Therefore, in the present experiment both virus and India ink were injected into the skin of 3 fasted animals and 2 controls As might have been expected from Experiment 1, the results (Table I, Experiment 2), indicate that the spread of India ink was considerably less in the fasted animals than that in the controls In the latter the mean area of spread was 7 84 cm² as compared with 5 68 cm² in the fasted animals In the same group, the logarithm of the respective dilution of virus was 6 86 (dilution 1 7,244,000) for the controls and 5 64 (1 437,000) for the fasted animals It thus appears that the decreased spread in the fasted rabbits parallels the increase in resistance

Effect of Undernourishment with and without Increase in Interstitial Fluid—Since the chance of viral lesions is decreased by localizing the virus (5), it was thought likely that some, at least, of the above results might be due to a reduction in spread of virus rather than to a change of the susceptibility in the individual cell directly referable to undernourishment To determine this point a method was needed by which rabbits might be brought to a poorly nourished condition without any change in the spread of materials in the skin Taylor and Sprunt (12) have shown that the spread is reduced by an increase in interstitial water and aided by the opposite It was therefore possible that the failure of particulate matter to spread, as noted in the last experiment, was due to an increase in interstitial fluid If present, such an increase would have to be dependent on some factor other than decreased plasma proteins, for these were normal Regardless of the mechanism responsible for the accumulation of interstitial fluid, edema does not develop if the animal is deprived of water The observation was also made that increases in interstitial fluid can be prevented if the fasting animal is injected intraperitoneally with physiological saline solution It is beyond the scope of this paper to attempt

TABLE I

The Effect of Undernourishment on Susceptibility to Infection with Vaccinia (As Indicated by the 50 Per Cent Point)

	No of rabbits used	No. of injections of each dilution	Change in weight before vaccination	Log of 50 per cent point	Mean spread of India ink in 4 hrs.	Increased resistance over controls
			per cent		*sq cm*	*X*
Experiment 1						
Controls	3	21	−1 1	6 85	—	—
Fasted with water	3	21	−19 1	5 88	—	9
Experiment 2						
Controls	2	14	+4 4	6 86	7 84	—
Fasted with water	3	21	−11 7	5 64	5 68	11
Experiment 3						
Controls	3	21	−0 6	6 23	8 91	—
Fasted with water	2	14	−27 4	5 35	5 38	7
Fasted with saline injected	1	7	−18 6	6 07	—	1
Experiment 4						
Controls	2	14	−1 8	8 86	8 63	—
Incompletely fasted with water	2	14	−17 2	8 44	7 51	3
Fasted with saline injected	2	14	−24 4	8 86	8 71	0
Experiment 5						
Controls	3	21	−2 8	8 07	7 38	—
Fasted with water	1	7	−18 5	6 67	5 38	12
Fasted without water	3	21	−22 6	7 98	—	0
Incompletely fasted with water	1	7	−20 0	8 14	7 40	0
Experiment 6						
Controls	3	21	−1 7	7 70	8 07	—
Fasted with water	1	7	−18 2	7 07	5 74	4
Fasted without water	2	14	−23 1	7 69	—	0
Incompletely fasted with water	2	14	−20 3	7 56	—	1
Experiment 7						
Controls	2	14	−2 5	7 85	7 27	—
Fasted with water	1	7	−16 0	7 06	7 93	6
Fasted without water	2	14	−29 4	7 75	—	0
Incompletely fasted with water	1	7	−17 6	7 71	7 41	0
Fasted with saline injected	3	21	−21 5	7 45	—	2

to explain these peculiarities in the metabolism of the rabbit. A series of experiments (Nos 3 to 7) was undertaken in which, by utilization of these two facts, it was possible to determine the effects of increased interstitial fluids and of simple undernourishment and to relate the former with the spread of India ink

The results of these experiments are shown in Table I This table shows conclusively that the fasted animals with increased interstitial fluid required from 3 to 12 times more virus to infect than did the controls Moreover the spread of India ink was also reduced This reduction of spread was certainly associated with, if not caused by, accumulation of excessive water in the tissues, for it was observed that the animals in this group (with the exception of those in Experiment 3) always lost less weight than the ones which were deprived of water or given physiological saline solution intraperitoneally This difference is apparently due to the fact that, if allowed to drink water, the fasted rabbits store it in the interstitial spaces

If the animals were deprived of both food and water (dehydrated), essentially the same amount of virus was needed to infect as in the controls The same was true in the case of those animals which received intraperitoneal injections of isotonic saline solution Unfortunately, in all but one instance, in which the spread was slightly larger than in the controls, measurements of the India ink spread in these 2 groups were unsatisfactory, and correlation of susceptibility with spread of particulate matter is therefore impossible However, in both groups, the blebs raised by the injection of the India ink suspension disappeared very rapidly, most of the ink remaining near the point of injection The identical behavior of the blebs in these 2 groups indicates that dehydration of the interstitial tissues characterizes both groups Apparently, the tissues were so dry that they allowed the menstruum of the ink to pass easily through the skin, while the large ink particles remained where they were injected Experiments are now under way to determine the amount of interstitial water by direct measurement

In addition to the above changes it was also noted that the animals deprived of both food and water and those that received physiological saline solution (dehydrated) had lesions which were relatively much smaller and which appeared about 24 hours later Their lesions regressed somewhat sooner than did those in the controls The lesions in the rabbits which received no food but were given water (increased interstitial fluid) were also smaller than in the controls but this difference was not as marked as with the above groups

In other words, depriving rabbits of food increases the resistance of the rabbit to infection with vaccinia, this resistance is accentuated by increase of interstitial fluids, and is offset by dehydration of the interstitial spaces

DISCUSSION

These experiments show that the response of rabbits to viral infection is affected in two ways by undernourishment One is the depletion of the necessary intracellular elements for virus multiplication As pointed out earlier in this paper, a susceptible cell must contain, regardless of its other requirements, the necessary constituents for virus multiplication At present little is

known as to what these requirements are, but Hoagland and associates (13) have shown that biotin is always present in purified vaccinia and is probably a necessary constituent of the virus Other substances of which the body has been depleted by undernourishment are probably equally necessary for virus multiplication According to this concept increased resistance to viral infection depends in some part at least on the absence of materials required for synthesis of the virus

The other influence is more striking and is associated with a change in the amount of interstitial fluids It has been shown previously in this laboratory (5, 12) that alteration in the amount of these fluids modifies the extent of spread of particulate matter in the tissues and that this spread can be correlated with the capacity of a virus to produce lesions Increase in interstitial fluids decreases the spread of particulate matter and reduces the incidence of viral lesions, while decrease in these fluids has the opposite effects It is quite likely that this relationship can be explained simply on the basis of number of cells exposed to the action of the virus, for as shown in this laboratory (5) and by Olitsky and Schlesinger (14), the larger the area of dissemination of a virus the greater the likelihood of its coming into contact with a susceptible cell and hence the greater the chance of a lesion The experiments reported here are entirely compatible with such an hypothesis However, an alternative possibility is that the susceptibility of the individual cell may be modified by changes in the amount of interstitial fluid. The whole question of the mechanism of water balance on susceptibility to infection is being studied further

It seems likely from the present experiments that both mechanisms are involved in determining the incidence of lesions Deprivation of food resulted in either fewer or smaller lesions. This effect was accentuated in the presence of accumulation of interstitial fluids but was partially nullified if the interstitial tissues were dehydrated

In this laboratory it has been observed that, generally speaking, rabbits do not react as well to infection with vaccinia in summer as in winter This may be the result of increased fluid in the tissues consequent upon changes in food and water intake in hot weather If this is so then experimental work with vaccinia and perhaps some other viruses might be facilitated in the summer by the injection of suitable amounts of isotonic salt solution

The possibility that invasion of the central nervous system by the virus of poliomyelitis may be aided by dehydration is also suggested De Rudder and Petersen (15) noted that poliomyelitis occurred among athletes in a boys' school after strenuous exercise and thought that the virus was already present and gained access to the nervous system because the nerve cells had been rendered more susceptible Perhaps the dehydration associated with the exercise may have increased the spread of the virus in the nervous tissue and thus led to infection

SUMMARY

Experiments are reported in which it is shown that if rabbits are deprived of food, the lesions resulting from injection of vaccinia are either fewer or smaller, presumably this is partially explainable on reduction of available nutrients in the cell The number and character of the lesions are also modified by the state of hydration of the interstitial tissues If the amount of interstitial fluid is increased by permitting the animal to drink water, the lesions are even less numerous, but if the interstitial tissues are dehydrated either by withholding water or by injecting physiological saline solution into the peritoneal cavity, then the lesions are more numerous

The increase in interstitial fluids in these experiments was not due to decreased plasma proteins, for these were normal In this respect, therefore, the rabbit differs from man, for unless the plasma proteins are reduced, simple starvation in man results in dehydration rather than edema of the tissues

From these experiments it is concluded that the virus is less able to multiply in the poorly nourished cell than in the well nourished one, and that hydration of the tissues increases the resistance of the tissue to infection while dehydration has the opposite effect It is suggested that this is because hydration tends to localize the virus *in situ*, with result that fewer cells are exposed to it, while dehydration has the opposite effect However, actual changes in cell susceptibility consequent upon altered water balance may be responsible for the effect

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THE REACTION OF PERIPHERAL BLOOD VESSELS TO ANGIOTONIN, RENIN, AND OTHER PRESSOR AGENTS*

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Plates 7 and 8

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One of the characteristics of the pressor substance which may cause essential hypertension in man or experimental renal hypertension in dogs must be an ability to elevate arterial pressure by constricting the arterioles sufficiently to increase peripheral resistance but not sufficiently to reduce blood flow Clinical experience indicates that patients or animals with hypertension do not have cold, pale skin indicative of reduced blood supply On the contrary, the plethoric appearance of many such patients is well known.

Experiments, both on animals and man, show that the ability of pressor substances to cause vasoconstriction varies greatly, not only in the same, but in different segments of the vascular system For example pitressin and adrenalin have in common the ability to reduce blood flow severely while the former elevates arterial pressure but little and the latter strongly As Landis, Montgomery, and Sparkman (1) pointed out, pressor agents which severely reduce blood flow to the periphery in relation to their ability to raise blood pressure, could scarcely be expected to be concerned in the genesis of hypertension characterized by normal or even elevated peripheral blood flow They found in rabbits that renin was unusual among the other pressor substances in elevating arterial pressure without consistent reduction of skin temperature,—which was accepted as a rough measure of peripheral blood flow

The demonstration that renin itself was not a pressor substance but produced elevation of pressure only after interaction with renin-activator and the liberation of angiotonin (2, 3) required that it also be shown that purified angiotonin elevates arterial pressure without significant fall in peripheral blood flow

Corcoran and Page (4) found no consistent change in skin temperature of trained normal dogs when angiotonin was infused. Both renin and angiotonin

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Except for such small changes in diameter, the venules did not react visibly to renin

Capillaries —Although the walls could be seen very clearly, no active constriction was observed when renin was injected, the lumens remaining open at all times Renin did not cause any increase in sticking of leukocytes to the walls, such as described by Clark and Clark (11) It did not produce any concentration of corpuscles, such as occurs following marked increase in capillary permeability, with loss of fluid into the surrounding tissue Nor did it cause diapedesis, hemorrhage, or visible endothelial injury

Reaction of the Vessels to Angiotonin

Forty injections of angiotonin were made, and three different preparations used

Arterioles —Angiotonin always caused arteriolar constriction As with renin, such constriction began in different arterioles at slightly different times, but in all started between 0 7 and 1 0 minute after the beginning of the injections Maximal constriction was reached within 1 0 to 1 5 minutes following the injections, and the arterioles returned to their original diameters within 2 0 to 11 0 minutes

The following example illustrates the typical response of an arteriole 28 microns in diameter to 0 2 cc of angiotonin Constriction began 1 0 minute after the start of the injection, and became maximal 1 4 minutes following the injection Three minutes after the injection the arteriole had returned to its control diameter Fig 14 shows arteriolar constriction produced by 0 2 cc of angiotonin

Increasing the amount of angiotonin injected caused greater arteriolar constriction The increase in constriction, however, was not proportional to the increased amounts of angiotonin injected Thus 0 25 cc of angiotonin caused an arteriole 31 microns in diameter to constrict to 0 88, 0 5 cc to 0 77, and 1 0 cc to 0 67 its original diameter The effect of increasing the amount of angiotonin from 1 0 to 2 0 cc is shown by another experiment One cc of angiotonin caused an arteriole 30 microns in diameter to constrict to 0 5 its original size (*cf* Fig 9), and 2 0 cc. to only 0 42 (*cf* Fig 10) Even the injection of 2 0 cc of angiotonin did not cause constriction of sufficient degree to stop the blood flow The response of the small arterioles to angiotonin was as great and in some instances greater than that of the larger ones

The arteriolar constriction produced by small amounts of angiotonin (0 2 cc) was not followed, after return of the arterioles to their control diameters, by dilatation as was the case with larger injections For example, the injection of 1.5 cc of angiotonin caused an arteriole 35 microns in diameter to constrict to 0 5 its original diameter This arteriole returned to its control diameter

5 0 minutes following the injection. Within 8.3 minutes it had increased to 1.3 its original diameter, and this dilatation lasted until 16.3 minutes after the injection

The walls of the arterioles became thicker when they constricted in response to angiotonin (*cf* Figs 5, 9, 10, and 14), indicating that such narrowing was not secondary to constriction of larger arterioles outside of the chambers

Venules —Angiotonin in amounts up to 0.5 cc. did not produce any visible effect upon the venules When the amount injected was increased to 1 0 cc., however, it caused, in most instances, a slight venular constriction. Thus 1 0 cc. of angiotonin caused a venule having a control diameter of 28 microns to constrict to 0 86 its original diameter

The degree of constriction of the venules was somewhat greater with greater amounts of angiotonin Thus, while in the above experiment, 1 0 cc. caused the venule to constrict to 0 86 its original diameter, 2 0 cc. caused a similar venule to constrict to 0 67 its original diameter

Except for such changes in diameter, the venules did not respond visibly to angiotonin

Capillaries —The capillaries did not react visibly to angiotonin No capillary constriction was observed, the lumens of the capillaries remaining open at all times (Figs. 6 and 7)

Reaction of the Vessels to Tyramine and Methylguanidine Sulfate

Tyramine and methylguanidine always caused arteriolar constriction when doses of 1.5 mg of the former and 7 0 mg of the latter were injected The constriction became maximal in 0.8 to 1.5 minutes with tyramine, and 0.8 to 1 2 with methylguanidine. Return to their original diameters occurred in about the same time (3 0 to 7.0 minutes) The degree of maximal constriction varied from 0 33 to 0.87 of the control diameters for tyramine and from 0.30 to 0 82 for methylguanidine Isopressor amounts of both tyramine and methylguanidine, usually, but not invariably, produced greater vasoconstriction than angiotonin (compare the arteriolar constriction shown in Fig 14 with that in Figs 16 and 18)

Tyramine and methylguanidine usually did not cause constriction of the venules in the amounts injected. In one of 5 experiments slight venular constriction was observed with both tryamine and methylguanidine.

No response of the capillaries to either substance was observed.

Reaction of the Vessels to Epinephrine and Pitressin

Epinephrine and pitressin always caused arteriolar constriction when doses of 0 0015 mg to 0 10 mg of the former and 0 6 unit to 1 unit of the latter were

injected Constriction became maximal in 0 5 to 2 0 minutes with both substances

Amounts of epinephrine and pitressin isopressor with 0 2 cc of angiotonin caused much greater arteriolar constriction (compare the arteriolar constriction shown in Figs 12 and 20 with that in Fig 14) At no time did the injection of 0 2 cc of angiotonin cause constriction to the point of interruption of the circulation, whereas constriction to this extent occurred in 4 out of 7 injections of the isopressor amount of epinephrine, and in 5 out of 6 injections of the isopressor amount of pitressin

Both epinephrine and pitressin caused constriction of longer duration than that produced by the isopressor amount of angiotonin Following the injection of 0 2 cc of angiotonin the arterioles returned to their original diameters within approximately 3 minutes, following 0 003 mg of epinephrine not until 10 minutes, and after ¾ unit of pitressin not until longer than 24 minutes

As with angiotonin the constriction of the small arterioles was as great proportionately as that of larger ones, and in some cases greater

In contrast to the effect of increasing the amount of angiotonin above 0 2 cc, increasing the amount of epinephrine above 0 003 mg caused much more vigorous and prolonged constriction Although this is probably also true of pitressin, the data are insufficient

As compared with the arteriolar constriction produced by isopressor amounts of tyramine and methylguanidine, epinephrine and pitressin caused much more (compare the constriction shown in figs 12 and 20 with that in Figs 16 and 18)

Both epinephrine and pitressin caused constriction of the venules, the degree of constriction being greater with larger amounts, but not exceeding 0 33 the control diameter in any instance The degree of venular constriction was greater than that caused by the isopressor amount of either angiotonin, tyramine, or methylguanidine

A typical experiment with epinephrine demonstrates that the venular narrowing was not due to arteriolar narrowing, but was an active constriction In this instance, 0 0025 mg of epinephrine caused arteriolar constriction which stopped the blood flow for 4 3 minutes During this time the venules did not become narrower Venular constriction began after the arterioles had begun to relax and the blood flow had started

Neither epinephrine nor pitressin caused active capillary constriction, the lumens of the capillaries remaining open at all times Following injections which stopped the circulation for 5 minutes or longer by constriction of the arterioles, sticking of leukocytes to the walls of the capillaries increased from phase 1 to phase 2 of the Clark and Clark (11) scale Otherwise no changes were noted

DISCUSSION

The evidence obtained by direct observation of the effects of renin and angiotonin on the blood vessels of normal rabbit's ears indicates that in moderate doses they produce active vasoconstriction in the arterioles, and in large doses slight constriction of the venules besides The capillaries remain unaffected The blood flow is maintained The action of angiotonin was indistinguishable from that of renin except maximal constriction of the arterioles occurred earlier with angiotonin and relaxation was more rapid Methyl guanidine sulfate and tyramine have somewhat similar actions All of these pressor agents contrast sharply in their action with epinephrine and pitressin, which cause severe vasoconstriction and reduction of blood flow in amounts which elicit similar rises in arterial pressure

This suggests that the elevated arterial pressure with maintenance of peripheral blood flow caused by administration of angiotonin, renin, tyramine, and methylguanidine is due to their action on the heart as well as on the arterioles. Augmentation of the force of the heart beat by direct action on the myocardium, increase in the rate of filling, or both, would account for the increased action of the heart Direct action of angiotonin on the heart is strongly suggested by the perfusion experiments of Hill and Andrus (12) and Lorber and Visscher (13)

In rabbits large doses of angiotonin are required to produce active venular constriction The rise in venous pressure would aid in increasing return of blood to the heart. In human beings the rise in venous pressure is marked, and decrease in vital capacity occurs (Wilkins and Duncan (6)), suggesting that in man the action on the venules may be more marked than in rabbit's ears However, the action on large veins with more developed muscle may be greater in rabbits than that on the relatively small venules studied

The renal vessels appear to be more sensitive to the vasoconstrictor action of angiotonin than the vessels of the ear This is suggested by the fact that even with moderate elevation of arterial pressure Corcoran and Page (4) found marked renal vasoconstriction and reduction of blood flow when either renin or angiotonin was injected Pitressin, on the other hand, has a much more variable effect on the renal circulation, sometimes increasing and sometimes decreasing it, while always eliciting severe vasoconstriction in the peripheral vessels.

The evidence is clear both from clinical observation of hypertensive patients and from Kapp, Friedland, and Landis' (14) study of the effect of experimentally induced hypertension in rabbits on skin temperature, that blood flow in the peripheral tissues is not significantly reduced Angiotonin in contrast with epinephrine and pitressin can produce hypertension without reduction of peripheral blood flow

The observations made in this investigation may be added to those which strengthen the view (15) that the renin-angiotonin vasopressor system has characteristics consistent with those required of a substance or substances concerned in the genesis of chronic arterial hypertension

SUMMARY

1 Both renin and angiotonin in small doses cause constriction of the arterioles in the ears of normal rabbits, as seen directly with the microscope Capillaries appear unaffected while venules exhibit slight or no constriction with small doses and moderate constriction with large doses The flow of blood through the tissues is not reduced except when very large doses are administered Tyramine and methylguanidine sulfate in isopressor amounts act somewhat similarly

2 Isopressor amounts of epinephrine and pitressin, by contrast, elicit severe vasoconstriction of arterioles lasting longer than that due to angiotonin, and flow of blood is sharply reduced or abolished altogether The degree of venular constriction was also greater, while the capillaries remained unaffected

3 The vasoconstrictor action of angiotonin on peripheral vessels in moat chambers in normal rabbit's ears is indistinguishable from that of renin, except that it is more rapid

CONCLUSIONS

As judged by study of the blood vessels in moat chambers in the ears of normal rabbits, angiotonin and renin elevate arterial pressure with little or no reduction of blood flow by causing constriction of arterioles and augmenting the force of the heart beat Tyramine and methylguandidine sulfate act somewhat similarly In contrast, epinephrine and pitressin in isopressor amounts elevate arterial pressure by causing arteriolar vasoconstriction, but the constriction is severe enough to reduce or abolish blood flow Angiotonin, unlike epinephrine or pitressin, acts on blood vessels of the rabbit's ear in a manner not inconsistent with that required of a substance capable of producing chronic hypertension

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EXPLANATION OF PLATES

PLATE 7

FIG 1 Photomicrograph showing the normal appearance of a living arteriole and venule in the bay of a moat chamber, 28 minutes before the intravenous injection of 0 2 cc of renin The grey blur in the lumens of the vessels is caused by circulation of the blood. The arrows indicate the direction of blood flow *A*, arteriole, *V*, venule × 168

FIG 2 Photomicrograph of the same vessels shown in Fig 1, 2 5 minutes following the injection of 0 2 cc renin This was the time of maximal arteriolar constriction produced by the renin The grey blur in the lumens of the vessels shows that the flow of blood through them did not stop As shown in this photomicrograph, the arteriolar wall became thicker during constriction × 168

FIG 3 Photomicrograph of the same vessels shown in Figs 1 and 2, 2 5 minutes following the injection of 0 2 cc Ringer's solution This injection did not cause arteriolar constriction (compare with Figs 1 and 2) × 168

FIG 4 Photomicrograph showing the normal appearance of a branching arteriole and a venule in the bay of a moat chamber, 26 minutes before the intravenous injection of 1 0 cc of angiotonin The arrows indicate the direction of blood flow *A*, arteriole, *V*, venule ×337

FIG 5 Photomicrograph of the same vessels shown in Fig 4, 1 0 minute following the injection of 1 0 cc of angiotonin This was the time of maximal arteriolar constriction produced by the angiotonin As shown in this photomicrograph, the arteriolar wall became thicker during constriction The flow of blood was not interrupted × 337

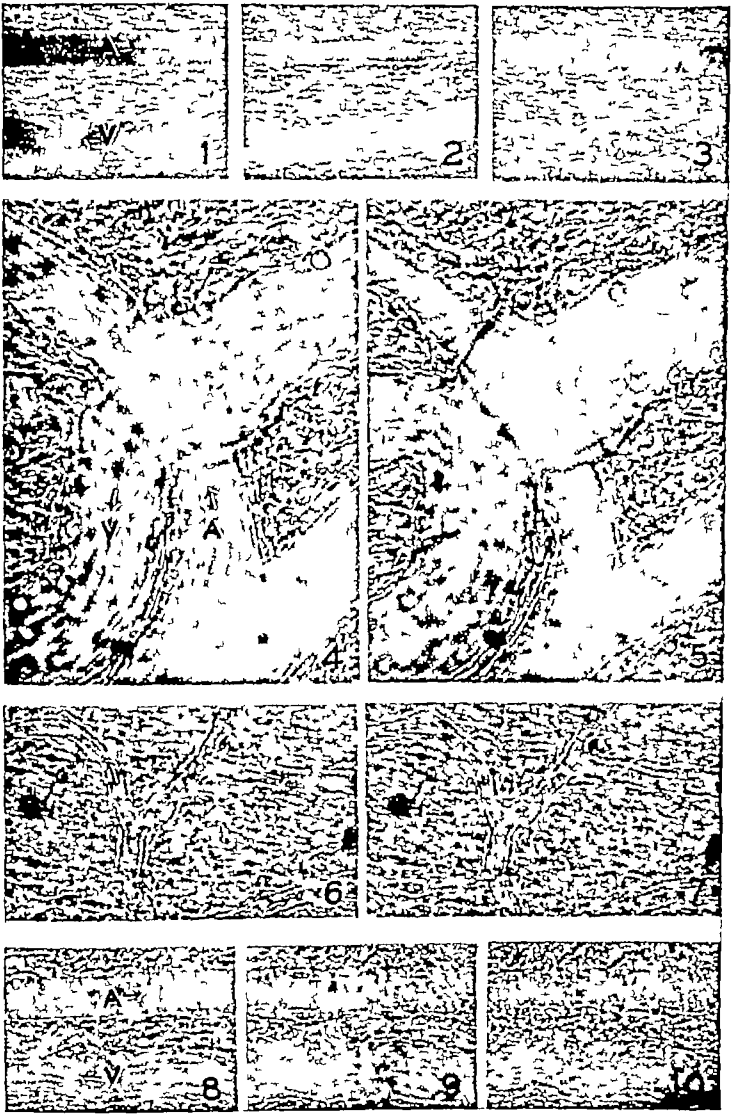
FIG 6 Photomicrograph of a capillary plexus in the same chamber as the vessels shown in Figs 4 and 5, taken 6 minutes before the injection of 1 0 cc of angiotonin This plexus received its blood from one of the terminal ramifications of the left branch of the arteriole shown in Figs 4 and 5 The walls of the capillaries can be seen, and the blur in their lumens indicates blood flow, the direction of which is shown by the arrows The actual diameter of these capillaries was approximately 9 microns × 337

FIG 7 Photomicrograph of the same capillary plexus shown in Fig 6, taken 1 0 minute following the injection of 1 0 cc of angiotonin This was the time of maximal arteriolar constriction (*cf* Fig 5) The flow of blood in the capillaries was not stopped, as shown by the blur in their lumens, and the capillaries did not constrict × 337

FIG 8 Photomicrograph showing the normal appearance of an arteriole and venule in the bay of a moat chamber, 42 minutes before the injection of 1 0 cc. of angiotonin The direction of blood flow is shown by the arrows *A*, arteriole, *V*, venule × 230

FIG 9 Photomicrograph of the same vessels shown in Fig 8, 0 9 minute after the injection of 1 0 cc of angiotonin The photomicrograph shows the maximal degree of arteriolar constriction produced by this injection The flow of blood was not stopped. × 230

FIG 10 Photomicrograph of the same vessels shown in Figs 8 and 9, 1 6 minutes following the injection of 2 0 cc. of angiotonin. The photomicrograph shows the maximal degree of arteriolar constriction produced by the angiotonin. The flow of blood was not stopped, and the arteriolar constriction was not proportionately greater than that produced by 1 0 cc. of angiotonin (compare with Fig 9) × 230



(Abell and Page Reaction of blood vessels to p

PLATE 8

FIGS 11 to 20 Photomicrographs of living arterioles and venules in the bay of a moat chamber, showing the effect upon the vessels and the circulation of the intravenous injection of isopressor amounts of epinephrine, angiotonin, tyramine, methylguanidine sulfate, and pitressin Figs 11, 13, 15, 17, and 19 are controls for 12, 14 16, 18, and 20, respectively The latter photographs, *i e*, 12, 14, etc, were taken at the time of maximal arteriolar constriction in all instances *A* and A_1, arterioles, *V*, venule × 230

FIG 11 Normal appearance of the vessels 6 0 minutes before injection of 0 003 mg of epinephrine The arrows indicate the direction of blood flow

FIG 12 The same vessels 1 0 minute following the injection of 0 003 mg of epinephrine The arterioles have constricted to the point of stopping the blood flow Stationary erythrocytes, in rouleaux formation, can be seen within the venule

FIG 13 The same vessels 21 minutes following the above injection of epinephrine The arterioles are slightly dilated, due to the epinephrine This photomicrograph was taken 7 0 minutes before injection of 0 2 cc of angiotonin

FIG 14 The same vessels 1 2 minutes following the injection of 0 2 cc of angiotonin The arterioles have constricted, but not to the point of stopping the blood flow

FIG 15 The same vessels 18 minutes after the above injection of angiotonin This photomicrograph was taken 4 minutes before the injection of 1 5 mg of tyramine

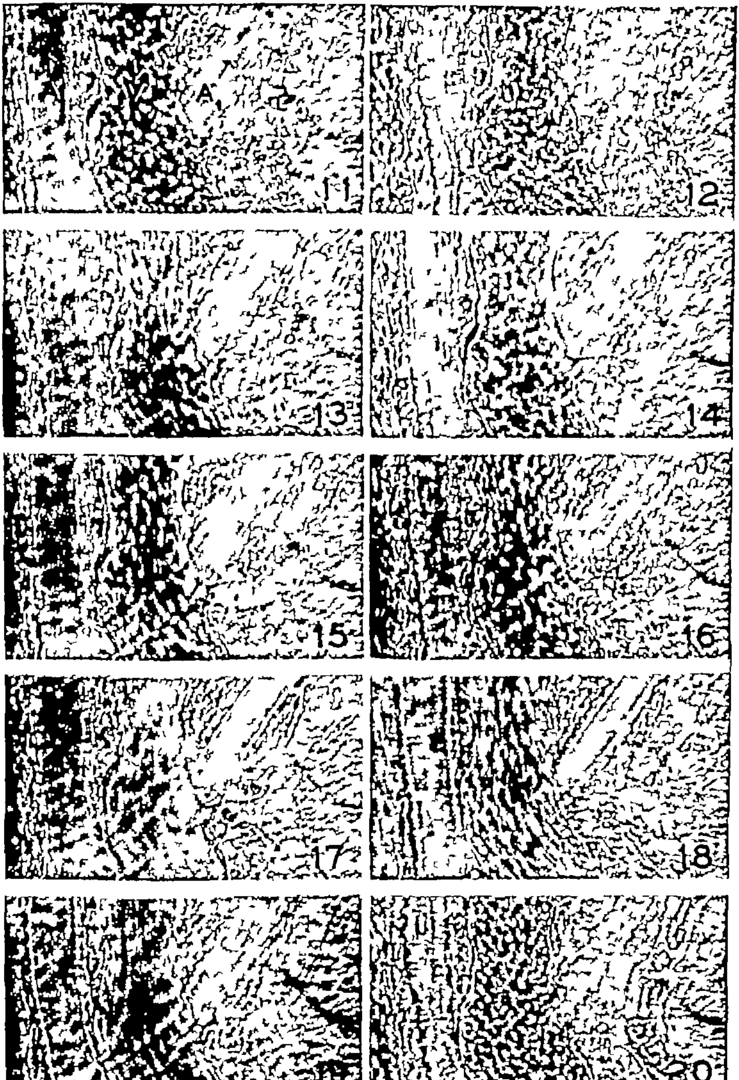
FIG 16 The same vessels 0 7 minute after the injection of 1 5 mg of tyramine The arterioles have constricted, but not to the point of stopping the circulation

FIG 17 The same vessels 23 5 minutes after the above injection of tyramine This photomicrograph was taken 4 minutes before the injection of 7 0 mg of methylguanidine sulfate

FIG 18 The same vessels 1 5 minutes after the injection of 7 0 mg of methylguanidine The arterioles have constricted, but not to the point of interrupting the blood flow

FIG 19 The same vessels 27 0 minutes following the above injection of methylguanidine This photomicrograph was taken 8 5 minutes before the injection of ¾ unit of pitressin

FIG 20 The same vessels 1 1 minutes after the injection of ¾ unit of pitressin The arterioles have constricted to the point of stopping the circulation Stationary erythrocytes, in rouleaux formation, can be seen within the venule



(Abell and Page Reaction of blood vessels to pressor agents)

HUMAN ALLERGY TO MAMMALIAN SERA

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In a previous communication report was made of a study of twenty two patients found to be allergic to one or more mammalian sera (1) The findings there reported may be summarized as follows Skin tests (scratch method) were performed on these patients with seven to fourteen mammalian sera, namely those of the horse, cow, dog, guinea pig, hog, rat, rabbit mouse, sheep, cat, porpoise, elephant, opossum, and monkey The tests gave the following results Seven patients were sensitive to horse serum but not to any of the other sera with which they were tested Seven were sensitive, to some degree, to all the mammalian sera with which they were tested Three were sensitive, to some degree, to all the mammalian sera with which they were tested except one Several were sensitive to two three, or four sera but not to the others with which they were tested In Table I are shown the results of tests on the four patients included in the present study

Skin tests performed with serial dilutions of sera on those patients who were sensitive to several or all the mammalian sera with which they were tested indicated that the degrees of hypersensitiveness to the various sera varied considerably in some patients while in others these degrees of hypersensitive ness were remarkably similar Local passive transfer tests indicated that positive skin reactions to the various sera were often but not invariably ac companied by transferable reagins

In those cases in which there were reactions to only one serum the hyper sensitiveness is apparently species-specific In other cases, however in which there were reactions to several or all the sera with which tests were made, several questions arise Is the hypersensitiveness in these cases also species-specific? Did these patients become sensitized to these various sera by ex posure to each serum individually or is it possible that exposure to one mam malian serum may result in hypersensitiveness not only to that particular serum but also to several or many other mammalian sera? These questions are particularly applicable to those cases in which there were positive tests to the serum of animals such as the guinea pig, opossum, porpoise and elephant—animals with which these patients had, in all probability, no previous contact either by way of respiratory tract, alimentary tract, skin, or parenteral in

TABLE I

Direct Skin Reactions on Patients

Scratch method Sera diluted with equal volume of glycerine

Case No	Clinical diagnosis	Horse dander	Serum of: Horse	Cow	Dog	Guinea pig	Hog	Rat	Rabbit	Sheep	Cat	Porpoise	Frog	Chicken
16	Perennial hay fever and asthma	++++	++++	++++	++++	++++	++++	++++	++++		++++	++++	–	–
18	Seasonal hay fever	++	++++	–	++++	++++	++++	++++	++++		++++	++++	–	–
21	Seasonal hay fever	–	+++	+++	++	+	+	–	++	++++		+++	–	–
22	Asthma	–	++++	++++	++++	++++	++++	++++	++++	++++				–

+ = small reaction
++ = moderate reaction
+++ = large reaction
++++ = very large reaction
All gave negative reactions to human serum

jection A number of authors have reported crossed reactions to mammalian sera in experimental animals (5)

EXPERIMENTAL

In order to obtain further evidence studies were made on the serum of four patients by the method of *in vitro* neutralization of antibodies and subsequent local passive transfer, as described by Walzer (2) In order to prevent chemical contamination (mixing) of allergens special precautions were taken in the cleansing of glassware and when syringes were used for intradermal tests and antibody neutralization a new syringe and needle were set aside and used for each individual animal serum.

The reagin-bearing serum of the patient 0 50 cc. and the neutralizing animal serum, 0 50 cc. of 1–100 dilution, were drawn into a 1 00 cc tuberculin syringe thoroughly mixed, and refrigerated in the syringe until the following day This mixture was then injected intradermally, 0 10 cc into each of eight (or nine) skin sites into a person not allergic to any of the sera used. The sites were marked accurately with ink and at a later time, usually after 1 to 3 days each site was tested with a different serum 0 02 cc. intradermally, as indicated in Tables II to V The controls, as indicated in the tables, consisted of (1) chicken serum 1–100 dilution, 0 02 cc., injected into skin sites previously injected with a mixture of the patient's reagin bearing serum plus mammalian serum (2) the patient's serum plus chicken serum, refrigerated and injected into skin sites which were later tested with the various mammalian sera and also with chicken serum (3) the various mammalian sera, 1–100 dilution 0 02 cc., injected into previously uninjected (normal) skin sites of the same recipient on whom the tests were being done.

Only one mammalian serum reagin bearing serum mixture was injected into a given recipient on the same day in order to guard against the possibility of one mammalian serum diffusing from its site of injection and neutralizing antibodies at some other injection site—antibodies which had previously been mixed with some other mammalian serum The possibility of neutralization of anibodies by allergens absorbed from the alimentary tract was considered but it was found that the eating of cooked beef (chiefly striated muscle) between the time of injections of the reagin bearing serum and subsequent skin test did not interfere with the local passive transfer reaction to cow serum.

Experiments involving *in vitro* neutralization and passive transfer of reagins are subject to certain difficulties, among which were noted

1 Certain persons are poor recipients, *i.e*, they do not readily accept local passive transfer of hypersensitiveness.

2 The site of passive transfer may be partially refractory for several days after injection of the reagin-bearing serum.

3 Excess allergen, from the allergen reagin mixture, may be absorbed from the injection site and may neutralize reagins at a distant skin site, including those of the positive controls, which, in these experiments were placed in the opposite arm

4 Scrupulous cleanliness of glassware must be observed in order to prevent mixing of allergens. It is best to reserve a separate syringe for each allergen

TABLE II

Antibody Neutralization, Case 16

Serum of patient plus serum of	Incubated injected into recipient, skin site tested with serum of								
	Horse	Cow	Dog	Guinea pig	Hog	Rat	Rabbit	Sheep	Chicken
Horse	−	−	++++	+++	−	−	++	++	−
Chicken	++	+	++++	+++	±	+	++	++	−
Cow	++++	−	++++	++++	+++	+++	+++	+++	−
Chicken	++++	++	++++	++++	+++	+++	+++	+++	−
Dog	−	−	−	−	−	−	−	−	−
Chicken	+++	+	++++	++	+	+	++	++	−
Guinea pig	++++	++	++++	−	−	−	+++	++	−
Chicken	++++	++	++++	++	+	−	+++	++	−
Hog	++++	−	++++	+++	−	++	+++	+++	−
Chicken	++++	++	++++	+++	+	++	+++	+++	−
Rat	++++	+	++++	+	++	−	+++	++	−
Chicken	++++	++	++++	+++	++	++	+++	++	−
Rabbit	++	−	++++	++	+	+	−	++	−
Chicken	+++	−	++++	++	+	++	+	++	−
Sheep	++++	−	+++	+++	+	+	+++	−	−
Chicken	++++	+	++++	++++	+++	++	+++	++	−

TABLE III

Antibody Neutralization, Case 22

Serum of patient plus serum of	Incubated injected into recipient, skin site tested with serum of								
	Horse	Cow	Dog	Guinea pig	Hog	Rat	Rabbit	Sheep	Chicken
Horse	−	±	+++	+	+	−	++	++	−
Chicken	++	±	++++	++	++	++	++	++	−
Cow	++++	−	++++	++++	+++	+++	++	+++	−
Chicken	++++	+	++++	+++	+++	+++	+++	+++	−
Dog	−	−	−	−	−	−	−	−	−
Chicken	+	±	++++	++	+	+	++	+	−
Guinea pig	+	−	++++	−	±	−	+	+	−
Chicken	++	±	++++	++	++	++	++	+++	−
Hog	+++	+	++++	++	−	−	++	++	−
Chicken	++++	+	++++	++	+	+++	+++	++	−
Rat	++	−	++++	+	−	−	+	+	−
Chicken	+++	±	++++	++	++	+++	++	++	−
Rabbit	++	±	+++	+	−	−	−	±	−
Chicken	++	±	+++	++	+	+	++	++	−
Sheep	+++	−	++++	+	±	−	+	−	−
Chicken	+++	−	++++	+++	++	++	++	++	−

TABLE IV

Antibody Neutralization, Case 21

Serum of patient plus serum of	Incubated, injected into recipient, skin site tested with serum of							
	Horse	Cow	Guinea pig	Hog	Rat	Rabbit	Sheep	Chicken
Horse	−	++	−	−	−	−	+++	−
Chicken	+	++	+	+	−	++	+++	−
Cow	−	−	−	−	−	−	+++	−
Chicken	+	++	+	+	−	−	+++	−
Guinea pig	−	+	−	−	−	−	++++	−
Chicken	±	++	±	±	−	±	++++	−
Hog	±	+	−	−	−	−	+++	−
Chicken	+	++	−	±	−	+	+++	−
Rat	Patient not sensitive to rat serum							
Chicken								
Rabbit	+	++	−	−	−	−	++++	−
Chicken	+	++	−	−	−	+	++++	−
Sheep	−	−	−	±	−	−	−	−
Chicken	++	+++	+	+	−	+	++++	−

TABLE V

Antibody Neutralization Case 18

Serum of patient plus serum of	Incubated, injected into recipient, skin site tested with serum of							
	Horse	Cow	Guinea pig	Hog	Rat	Rabbit	Sheep	Chicken
Horse	−	−	−	−	−	−	−	−
Chicken	++++	−	+++	++	++	++	+	−
Cow	Patient not sensitive to cow serum							
Chicken								
Guinea pig	++++	−	−	−	−	++	−	−
Chicken	++++	−	+++	++	++	+++	+++	−
Hog	+++	−	+++	−	−	++	±	−
Chicken	++++	−	+++	++	++	+++	++	−
Rat	++++	−	+	−	−	−	+	−
Chicken	++++	−	+++	+++	++	++	++	−
Rabbit	++++	−	−	−	−	−	−	−
Chicken	++++	−	++	++	++	++	+	−
Sheep	+++	−	±	−	−	+	−	−
Chicken	++++	−	+++	++	++	++	++	−

For these and other reasons it was often necessary to repeat the transfers several times in order to obtain satisfactory results The results of many experiments had to be discarded entirely When precautions are observed and the proper controls carried out, however, it is believed that the results are reliable and warrant certain deductions

FINDINGS

As indicated in Tables II to V the hypersensitiveness was partially species-specific as proven by the fact that, in each case, one particular mammalian serum (dog in cases 16 and 22, horse in No 18, sheep in No 21) neutralized reagins for all mammalian sera with which tests were made, whereas the other mammalian sera neutralized reagins for themselves alone or for themselves plus one or several additional sera but not for all sera The ability of any particular mammalian serum to neutralize reagins for other mammalian sera varied with the different reagin-bearing sera, thus indicating that the reagins for any one particular mammalian serum differed in different individual patients

DISCUSSION

The neutralization by one allergen of antibodies for another allergen may be explained in three ways (1) By assuming that antibodies are not absolutely specific but may react not only with the allergen which stimulated their production but also with other allergens having a closely related chemical structure Evidence that this is one correct interpretation has been presented by Landsteiner working with artificial, conjugated compounds (5) (2) By assuming that immunologically related substances, such as mammalian sera, are mixtures of two or more allergens and contain one or more of these allergens in common (3) By assuming that a single molecule may contain multiple allergenic determinants and that different chemical compounds obtained from different sources contain one or more of these determinants in common, as suggested by the work of Hooker and Boyd (6)

In several of the cases reported here it will be observed that antibodies for a given mammalian serum, obtained from one patient, were neutralized by several other mammalian sera, whereas antibodies for the same serum, obtained from another patient, were not entirely neutralized by the same sera This fact definitely proves that the antibodies for this particular mammalian serum obtained from two different sources are not identical These differences in antibodies may be partially explained by assuming that the antibodies were brought into existence as the result of stimulation by different allergens, in one case, for example, by an injection of horse serum, in another by the eating of mutton, and in a third by the inhalation of dog dander, and so forth

Several of the original twenty-two patients included in the previous report were sensitive to only one of the sera with which they were tested Others

were sensitive to several sera in varying degree, the reactions ranging from strongly positive to certain sera to negative to one or more other sera The degrees of sensitivity to these different sera varied greatly in different patients, certain sera gave strongly positive reactions in some patients and negative reactions in others It follows that the (fixed tissue) antibodies in these patients are different. The absorption experiments described above have demonstrated that the antibodies, in some cases, are not entirely species-specific. These facts suggest that the antibodies for a given mammalian serum, obtained from different patients, are directed against (specific for) different allergenic elements in that serum

The fact that several of these patients were found to be sensitive to all the mammalian sera with which they were tested, except human serum, suggests the possibility of an allergen common to all mammalian sera except that of man This assumption logically calls for another, namely, the existence of an allergen common to all mammalian sera except that of the horse, etc. Thus each mammalian serum would be assumed to be characterized not only by a species-specific factor but also by the *absence* of an allergen common to all other mammalian sera, and the *presence* of innumerable common allergens —a conclusion which is very improbable, if not absurd This difficulty may be overcome, however, by assuming the existence of a relatively small number of allergenic complexes or determinants, present in the various mammalian sera in different numbers and combinations, certain of the complexes being absent in certain sera and present in other sera. One may suppose that *mammalian sera contain many allergenic elements Among these there is at least one for each species which is species-specific The others vary in their distribution among different species. Certain of these elements have a wide distribution, while others have a more limited distribution The serum of any one species of mammal is lacking in one or more of the elements which are present in the sera of some of the other species* One would have to assume that different human individuals react differently in their response to the same complex of allergenic stimuli (3) This assumption, however, is in agreement with the clinical fact that different individuals in the same environment, inhaling the same substances (pollens, for example) become sensitized to different substances or to the same substances in different degrees It is also in agreement with the experiment of Landsteiner, *et al* (4) (in another type of allergy) in which two chemicals, paranitrosodimethylaniline and dinitrochlorobenzene were applied to the skin of man Certain individuals became sensitized only to the former, others only to the latter, while others became sensitized to both but in varying degree to each

This hypothesis explains (1) positive reactions to many mammalian sera in persons who have probably had contact with only one or a few of these sera, (2) positive reactions to certain sera, such as those of the guinea pig, porpoise,

opossum, and elephant, contact with which (even through the medium of the respective danders) is very rare in the general population, (3) positive skin reactions to horse serum in persons in whom there is no history of contact with horses and no history of injection of horse serum preparations In such cases the hypersensitiveness may have resulted from the eating of (partially cooked) beef, pork, mutton, or other mammalian meat, or perhaps from the inhalation of the dander of the cat, dog, or other mammal This hypothesis does not, of course, exclude the possibility of species-specific sensitivity to several different mammalian sera in the same individual, nor does it deny the possibility that antibodies may react with substances closely related chemically to those which stimulated their production

SUMMARY

Four patients allergic to many mammalian sera were found to have circulating, skin-sensitizing antibodies for these sera A study of these antibodies by *in vitro* neutralization and subsequent local passive transfer showed that, in the case of each patient, one particular mammalian serum neutralized antibodies for all sera whereas other mammalian sera neutralized antibodies for themselves alone or for themselves plus one or several additional sera but not for all sera The possibility of multiple common allergenic determinants in mammalian sera is discussed

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STUDIES ON LYMPHOGRANULOMA VENEREUM

I DEVELOPMENT OF THE AGENT IN THE YOLK SAC OF THE CHICKEN EMBRYO

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PLATES 9 TO 11

(Received for publication, December 4 1941)

The demonstration that the method of yolk sac inoculation, first used so successfully by Cox (1) in his investigations on *Rickettsiae*, could be applied with success to the growth of the agent of lymphogranuloma venereum (2) has stimulated more extensive investigations of this disease and its causative agent (3–12) Amongst other studies which have been made is one of the nature of the development of the agent in the cells of the yolk sac. This has been the subject of a preliminary communication elsewhere (13) The purpose of the present paper is to describe this development in detail with regard both to morphological changes and to concomitant changes in the infective titre of the yolk sac

The first description of apparently specific inclusions in connection with lymphogranuloma venereum was given by Gamna in 1923 (14) and again in 1924 (15) when the observations were confirmed by Favre (16) Both these observers noted in mononuclear cells from infected lymph nodes, whether in the natural human disease or in experimental disease in guinea pigs, cytoplasmic inclusions varying in size from those which were barely visible to others 4.5μ in diameter These might be multiple in one cell, varied in size, and were often either vacuolated or contained an acidophilic center and basophilic periphery They were not found in other conditions or in normal lymph nodes. Similar bodies were seen in lymphogranuloma venereum by Todd (17) In publications in 1933 (18) and 1938 (19) Findlay cast doubt on the virus nature of these bodies because many of them give a positive reaction for thymonucleic acid, but in retrospect it seems probable that they do represent different stages in the development of the agent.

In 1927 Gay Prieto described small bodies which seemed to be specific (20) These appeared both within and between the monocytes surrounding the abscesses they were of 1μ or less in size, were mostly round, oval or semilunar and were metachromatic. Twelve or more might appear in the cytoplasm of a single cell. In 1933 Findlay (18) also described bodies similar to those of Gay Prieto These were found in human material and also in the experimental disease in monkeys, guinea pigs and mice They were from 1 to 4μ in diameter and were found in lymphocytes, in plasma

cells, or lying free They were basophilic and the smaller ones sometimes occurred in large masses

In 1935 Miyagawa and his associates described for the first time "granulo-corpuscles" about 0 3μ in diameter which resembled closely the elementary bodies of other viruses (21) These bodies were found in monocytes, leucocytes, and glial cells in the brains of intracerebrally infected monkeys They were solitary, in pairs, or in groups, did not give a Feulgen reaction, and were negative with Gram stain Similar bodies were found in all human material from cases of lymphogranuloma venereum In a subsequent paper (22) similar bodies were described in monocytes, glial cells, and neurones of intracerebrally infected mice In advanced lesions they increased markedly in number so that they came finally to fill the infected cell Although not mentioned in the text, the illustrations for both these papers suggest that the specific bodies may vary in size The presence of these bodies was soon confirmed by other investigators, for example Nauck and Malamos (23), who noted that they resembled closely the elementary bodies of psittacosis in their staining reactions with Giemsa stain In the preparations of these authors paired forms were common The elementary bodies often occupied vacuoles in the cell cytoplasm or were embedded in a (with Giemsa) reddish violet matrix

Several filtration studies with Elford gradocol membranes were carried out by various investigators and gave results in keeping with the microscopic findings of Miyagawa and his associates (21) Thus by such filtration Broom and Findlay arrived at a size of from 125 to 175mμ for the elementary bodies (24)

Differences in size of the virus bodies and in their staining reactions soon were noted (25, 26), and in 1938 Findlay and his colleagues suggested that the virus might undergo a developmental cycle (27) In the early lesions in intracerebrally infected mice large bodies were seen 0 4 to 0 7μ in diameter These stained bluish violet with Giemsa and were often irregular in shape, suggesting fission Intermediate forms between these and the reddish violet elementary bodies of 0 1 to 0 175μ were seen The large forms occasionally occurred massed together into plaques Malamos (28) also noted larger blue staining bodies in the early stages of the infection of tissue cultures of rabbit corneal epithelium These larger bodies increased in size and, while homogeneous at first, were later seen to contain elementary bodies These larger plaques became vacuolated and might break up into shreds and uneven fragments In a later study Findlay and his colleagues gave a more detailed description of the suggested cycle of development of the agent in the brains of infected mice (19) Following intracerebral inoculation no virus could be seen in smears for some hours The initial bodies which were seen were larger than elementary bodies Since dumb-bell like division forms were noted it appeared that these large bodies divided Compact masses of large bodies might result from which individual bodies might break off, enter other cells, and give rise to elementary bodies but no decision was reached as to whether the elementary bodies themselves divided, although pairs and short chains were noted When sufficiently numerous in the cytoplasm, the elementary bodies formed collections within vacuoles with definite limiting membranes, the membranes might eventually rupture and liberate the elementary bodies which could then infect new cells

Amongst others who have noted more than one form of the virus bodies are Schoen

(29), and Gey and Bang (30) Schoen (29) noted that the first bodies to be seen inside vacuoles in the ependymal cells of infected mouse brains at 48 hours were 2 to 3μ. By 4 days the bodies had become smaller and might be bacilliform Gey and Bang (30) used a tissue culture of fibroblasts from human thyroid and grew the virus for as long as 7 months After 7 days small vesicles were seen in the fibroblasts and these later increased in size. The small vesicles contained bodies about 1μ in diameter but the larger vesicles which occupied the whole cell, were filled with bodies 0 2μ in diameter which showed brownian movement No large inclusions other than the vesicles were seen The latter were thought to represent response of the cells to the presence of the virus.

Technique

All the studies of development described below have been carried out with one strain of lymphogranuloma venereum, namely that obtained originally through the courtesy of Dr W L Fleming and termed by us J H However, similar morphological elements have been seen in the other three strains with which we have worked, one of which was isolated by inoculation of human pus into the yolk sac

All inoculations have been made into the yolk sac of 6 day old chicken embryos (1) and have been of 1 ml. volume. Suspensions have been made by shaking infected yolk sacs for 30 minutes on a machine with sufficient beef heart infusion broth (pH 7 6) to produce a 10 per cent dilution These yolk sacs had been freed of most of their yolk by careful draining but had not been washed. Throughout the investigation the titre of the virus has remained almost unchanged both for embryos by the yolk sac route with an L_D 50 of 10^{-9}, and for mice by the intracerebral route with an L_D 50 of 1 to 416 In studying the development of the agent in the yolk cells, different dilutions of infected yolk sac have been used as inoculum as will appear below but a dilution of 10^{-1} or 5 $\times$ 10^{8} infective doses has given us the most significant results. In titrating the amounts of virus present in the yolk cells at different stages of development the yolk sac method of inoculation has been used and eggs surviving for 15 days after inoculation (*i.e* to the point of hatching) have been opened and examined for the presence of virus both by smear and, in many cases, by section The titres given are therefore those of persistent infection rather than mortality although in most cases these have corresponded. Of 368 eggs which were opened on the 15th day after infection and found to be alive, 16 or 4 4 per cent have been found by smear or section to be infected. In 567 eggs whether dead or alive found negative on smear 42 or 7 4 per cent have been found positive on section The yolk sac method has also been used in estimating the amount of virus present in embryonic tissues other than the yolk sac.

In the morphological studies use has been made both of smears or impressions and of sections. Smears and impressions are made by smearing or gently impressing on a clean glass slide a small fragment of yolk sac from which excess free yolk has been removed on a gauze sponge For Gram or Macchiavello stain these smears or impressions have been heat fixed. For Giemsa stain whether the slow or the rapid method be used they have been fixed in 95 per cent methyl alcohol. Material for

paraffin section has been fixed in Zenker's or Helly's fluid. After the former fixation staining has been carried out with eosin and methylene blue or with Giemsa. After fixation with Helly's fluid Noble's stain, as described by Yanamura and Meyer (31), has been employed.

RESULTS

As has been indicated elsewhere (2), during the earlier stages of infection after yolk sac inoculation, virus could be demonstrated, by intracerebral inoculation of mice, only in the yolk sac and to a lesser degree in the yolk. The brain, viscera, and chorio-allantois contained no virus demonstrable by this method. If the infection were permitted to progress until the embryo was moribund or dead and titration were made in the yolk sac results were obtained as shown in Table I. The titre of the original inoculum and the consequent period of survival of the embryos did not appear to be of any great importance as far as the distribution and titre of virus in the moribund or dead embryo were concerned. It will be noted that the yolk contained considerable amounts of virus but always less than that found in the yolk sac, while the embryo itself or its component tissues contained much less. It seemed clear from these results that the multiplication of the virus was occurring principally in the yolk sac and that the virus found in the other tissues of the embryo was due in all probability to invasion of the vessels in the highly vascularized yolk sac tissue with perhaps slight multiplication in favorable sites. The presence of the virus in the yolk was due to the rupture of the infected yolk cells which, as will appear below, were often completely filled with virus bodies. At no time has there been any evidence of multiplication in the yolk itself, and studies have shown that, far from multiplying in yolk removed from the egg, viable virus rapidly disappears and in most cases a starting titre of 10^6 to 10^8 infective units per ml. has fallen to zero in 48 hours.

TABLE I

	Average 50 per cent endpoint of infectivity	Outside limits of titre in different experiments
Yolk sac	$10^{-8.42}$	10^{-7} to 10^{-9}
Yolk	$10^{-7.0}$	10^{-6} to 10^{-8}
Chorio-allantois and amnion	$10^{-1.95}$	10^{-1} to 10^{-4}
Whole embryo less membranes	$10^{-2.77}$	10^{-1} to 10^{-4}
Brain	$10^{-1.24}$	10^{-1} to 10^{-3}
Skin and muscle	$10^{-2.59}$	$<10^{-2}$ to 10^{-4}
Lungs	$10^{-2.25}$	10^{-1} to 10^{-3}
Liver, kidney, and spleen	$10^{-3.75}$	$<10^{-2}$ to 10^{-5}

For the above reasons it seemed logical to confine our investigations of the development of the agent of lymphogranuloma venereum to the yolk sac of

the embryo The most informative results have been obtained with an in oculum of 5 × 10⁸ infective units, or 1 ml of a 1 in 10 dilution of a heavily infected yolk sac, and frequent killing of embryos thereafter for both titra tion and morphological studies Representative titration curves are shown in Chart 1 which also includes certain important data from the morphological studies Table II contains the pertinent data.

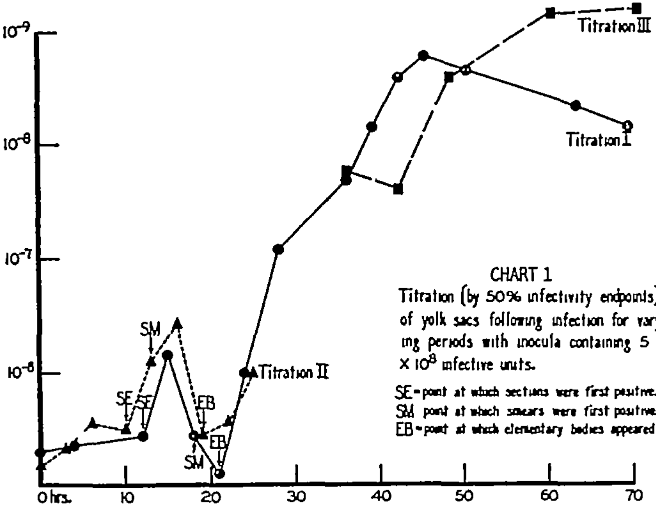

CHART 1

Titration (by 50% infectivity endpoints) of yolk sacs following infection for varying periods with inocula containing 5 × 10^8 infective units.

SE = point at which sections were first positive.
SM point at which smears were first positive.
EB = point at which elementary bodies appeared

In both of these studies large numbers of eggs were inoculated by the yolk sac method with 1 ml. of a 10^{-1} dilution of a heavily infected yolk sac. At successive short time intervals thereafter 3 eggs were opened and the yolk sacs removed. Small fragments of each yolk sac were used to prepare smears and impressions, larger frag ments were fixed in both Zenker's and Helly's fluids, and the remainders of the yolk sacs (approximately an equal amount from each of the 3 eggs) were pooled and shaken with sufficient beef heart infusion broth to give a 1 in 10 original dilution. From this dilution appropriate other dilutions were prepared and 10 eggs inoculated with each appropriate dilution by the yolk sac technique. On death of any of these test em bryos, or after 15 days incubation in the case of those surviving (*i.e* at hatching time) smears were made from the yolk sacs and these were stained with Giemsa and Macchiavello stains and examined for virus In those cases in which there remained any doubt after examination of the smears, sections were prepared and examined

As will be seen from the titrations there was but slight increase in the titre of virus up to 12 hours, during the next 4 hours however the rise in titre was rapid, being approximately tenfold This rise was followed by an abrupt fall to levels equal to those found at the commencement of the experiment A second rise began at about 22 hours and was more sustained than the early rise The maximal titre was reached in about 44 hours and later the titre leveled off or actually decreased slightly, this decrease being due presumably to the titration of less susceptible eggs which were the only ones to survive for 60 or more hours

TABLE II

50 Per Cent Endpoints of Infectivity

Titration I		Titration II	
Time from inoculation to testing of yolk sac	Result	Time from inoculation to testing of yolk sac	Result
hrs		*hrs*	
0	$10^{-5.29}$	0	$10^{-5.17}$
4	$10^{-5.36}$	3	$10^{-5.33}$
12	$10^{-5.44}$	6	$10^{-5.55}$
15	$10^{-6.16}$	10	$10^{-5.5}$
18	$10^{-5.43}$	13	$10^{-6.11}$
21	$10^{-5.11}$	16	$10^{-6.43}$
24	$10^{-6.0}$	19	$10^{-5.45}$
28	$10^{-7.09}$	22	$10^{-5.57}$
36	$10^{-7.71}$	25	$10^{-6.0}$
39	$10^{-8.17}$	Titration III	
42	$10^{-8.6}$		
45	$10^{-8.81}$	36	$10^{-7.77}$
63	$10^{-8.36}$	42	$10^{-7.62}$
69	$10^{-8.19}$	48	$10^{-8.59}$
		60	$10^{-9.21}$
		70	$10^{-9.25}$

Some indication of the morphological changes which accompanied these changes in titre are given in Chart 1 These studies of morphology were, as has been pointed out above, carried out both by smear and by section Up to the first 6 hours occasional virus bodies, elementary bodies, or others, could be seen in the smear or more rarely in sections From their staining reactions and morphological characters it was clear that these bodies represented virus of the original inoculum The period during which such bodies were found was always followed by a short period in which no virus could be observed in either section or smear, and this in turn was succeeded by the initial stages of the developmental cycle of the virus These initial stages were always observed first in section and later in smear and, for the sake of convenience, the changes in the former will be described first The picture of a cyclical de-

velopment which is given is open to the objection that it is obtained by the study of static preparations and must therefore be to some degree guesswork However, it may be emphasized that the picture is a composite one which has been obtained by the examination of several hundred yolk sacs in which tissue the virus is very plentiful and extremely easy to see Moreover the relationship of time to the stage of the development of the virus has been so precisely worked out that it is now possible to foretell with accuracy the picture which will be found in eggs opened at any given time after receiving a given number of infective units of virus

Following the disappearance of the originally inoculated virus, new virus bodies, which might or might not be surrounded by halos, appeared in the yolk cells where they lay close to the walls, at about 10 to 12 hours after original inoculation. Although in every instance the original inoculum consisted almost entirely of elementary bodies which showed after staining by the usual methods an average diameter of 400 mμ, the initial virus bodies seen in the yolk cells had a diameter of about 1μ or more than twice that of the original virus particles. Since these initial bodies when first seen were very scanty and consisted of single bodies or at most pairs, it seems probable that the earlier stages of the cycle were overlooked on account of the small size of the original infecting units. These appeared to make use of the first 10 to 12 hours to increase in size until they were readily visible (Fig 1) These initial bodies divided much like cocci to form pairs (Fig 2) tetrads, and small groups (Fig 3) By the time that these small groups (as in Fig 3) had begun to appear, definite structural arrangements might be seen The virus bodies appeared to be arranged within a vesicle with a limiting membrane which was more clearly defined in some instances than in others. The virus bodies might or might not be arranged close to the surface of this membrane and were already seen to be embedded in a thin matrix. Differential stains such as Noble s stain (31) showed that all of these virus bodies apparent at this time stained green with methylene green as did the thin matrix No virus which had retained the basic fuchsin and therefore stained red appeared until later Within a short time of the appearance of these small groups one began to see besides an augmentation in number of these initial bodies, a further definite increase in size (Fig 4) Thus by 16 hours bodies of 2μ in diameter were to be found and by 18 to 20 hours bodies 4μ in diameter were apparent. The matrix in which the bodies were embedded had become denser By this time, moreover definite structural differentiation might be seen within these larger bodies With eosin methylene blue stain or Giemsa, some of the bodies of between 2 and 4μ were seen to be vacuolated (Fig 5) while with Noble's stain at 18 hours a few of the larger green-staining masses were seen to contain one or more smaller muddy brown bodies which were undoubtedly bodies retaining the basic fuchsin seen through the dense green-staining surrounding material. By 20 hours definite bright red elementary bodies within large green bodies or plaques, or lying free were to be seen This is shown in Fig 6 in which a body about 3.5μ in diameter contains besides a small vacuole two small red bodies about 600 to 800 mμ in diameter embedded in the homogeneous dense green capsule. The plaques, already mentioned above, seemed to be produced by a steady augmentation in size of the larger bodies and only *rarely, if at all, by the fusion of two or more bodies*

Several such plaques might appear in one vesicle (Figs 7 and 8) They might reach a diameter of 4 5μ without showing any definite vacuolation when stained with eosin-methylene blue (Fig 9) but most of the bodies of 3μ or greater were markedly vacuolated (Figs 8, 10, and 11) Individual plaques might reach a diameter of at least 7μ (Fig 8) In most cases with eosin-methylene blue nothing could be seen in the vacuoles of the large bodies or plaques but occasionally, as in Fig 8, one might see quite definite little bodies of the size of elementary bodies in the vacuoles, and with the Noble stain many of the plaques were found to contain few or many red bodies of all sizes (Fig 12) However, after the examination of many preparations one is forced to the conclusion that the plaques when they become large and show vacuoles are very fragile and in most cases lose their content of elementary bodies during the handling necessary in the production of the fixed sections In Fig 11 it would appear that the central plaque, which is obviously disintegrating, has liberated large numbers of elementary bodies which in this case are still to be seen closely surrounding it

The changes seen in the first 18 to 20 hours may be summarized as follows The first bodies were seen in the yolk cells at 10 to 12 hours after inoculation and at this time were more than twice as large as elementary bodies During the next 6 hours these bodies increased in number (to 40 or more) and size (up to 4μ) and came to occupy small vesicles in which they were embedded in a thin matrix Between 18 and 20 hours the bodies increased still more in size and formed plaques which might be 7μ in diameter These latter showed vacuoles and with certain stains were seen to contain small granules of elementary body size By their disintegration elementary bodies were set free and the first cycle might be said to have been completed While therefore in many cases the first cycle might continue for 30 hours or longer, in some cases it took only 18 hours, this being the shortest period that we have noted It must be emphasized that all the diameters given are those of fixed and stained virus bodies

The liberated elementary bodies in many cases escaped from the affected cell and entered new cells to initiate new cycles of the same type as that described above

The first appearance of this new cycle, as heralded by single or paired initial bodies close to the walls of otherwise normal yolk cells, was at 28 to 30 hours, *i e* 10 to 12 hours after the "reinoculation" with new elementary bodies, thus confirming the 10 to 12 hour "silent" period noted in the first cycle If, however, they did not enter new undamaged cells, the history of the elementary bodies seemed to be different New small vesicles were formed in which the elementary bodies divided for the most part as such so that the vesicles came to contain enormous numbers of elementary bodies embedded in a thin matrix Just below the limiting membrane bodies of 1μ or greater might occur but they were less numerous than the elementary bodies Fig 13 to 16 show the development of these vesicles From small size (Fig 13) they came to occupy the whole cell (Fig 14) Later many cells are filled (Fig 15) and finally, at the point of death of the embryo, nearly all the cells are so affected (Fig 16) It is

difficult to distinguish individual elementary bodies in the central mass but larger bodies may be seen close to the periphery of the vesicles.

As has been pointed out above, virus appeared in the smears or impressions from 3 to 6 hours later than it did in the sections This was undoubtedly due to the fact that in smears the virus was derived by liberation from yolk cells damaged usually by the progress of infection and more rarely by the manipulation used in making the preparations In the early hours virus was too scanty in the cells, and cell damage was so slight, that no bodies appeared in the smears. Once virus did appear in these preparations however, it went through the same cycle as that seen in the sections

At about 14 hours one began to see single virus bodies and even small groups similar to that shown in Fig 17 The similarity of this to the group shown in Fig 3 (in a section) is immediately apparent Slightly later the groups were larger and by 20 hours were seen to contain bodies of the size of elementary bodies (Figs 18 and 19) With Macchiavello stain the majority of these bodies stained blue up to 18 hours but even at 13 hours red, large bodies were seen Later the proportion of red bodies to blue increased but at all times some blue bodies even of the size of elementary bodies were to be seen Intact plaques were not seen in smears and any plaques were rare, but every now and then plaques in the process of disintegration were seen (Fig 20) Scattered fragments of matrix were seen in the early stages, *i.e* at 13 hours, but later larger masses of matrix in the process of disintegration were to be seen (Figs 21 to 23) In Giemsa stains virus bodies could be seen in these masses, albeit with difficulty but with Macchiavello stain red or blue virus bodies stood out clearly against the pale blue of the matrix (Fig 23) In much later stages, *i.e* at 48 hours elementary bodies predominated but larger bodies were still to be seen The so called dumbbell forms described for psittacosis were seen and appeared to be forms undergoing division and separation (Fig 24) At the time of death elementary bodies formed by far the majority of forms seen (Fig 25) In some smears yolk cells containing large numbers of elementary bodies were seen (Fig 26) or others in which the masses of elementary bodies remained intact although dislodged from the yolk cell (Fig 27) Frequently one saw monocytes containing virus bodies (Fig 28) but this would appear to represent only phagocytosis of the virus particles A photograph (Fig 29) of colonies of staphylococci growing in the yolk cells is inserted for comparison of size.

With Noble's or Macchiavello's stain the majority of elementary bodies forming a new inoculum retained the basic fuchsin and stained bright red. However as has been pointed out above the initial bodies were invariably green with Noble s stain, and no red bodies were seen until about 18 hours when brownish red bodies (or red bodies apparently partly obscured in green capsular material) were seen. With Macchiavello's stain red initial bodies might appear in smears albeit rarely as early as 13 hours Eighteen hours is the time of the first appearance of elementary bodies and it might appear from this that the elementary bodies were the essential virus bodies, that they always retain the basic fuchsin when exposed to it but that in the early stages of the cycle they were concealed in a dense capsule which stained with methylene green (or blue) so that they could not be seen until the capsule became

larger and less dense That this simple explanation will not suffice, however, is shown by the fact that at no stage were all of the elementary bodies red (and this is particularly true with Macchiavello's stain), and furthermore that red bodies might occur of at least 2μ in diameter, that is, as large or larger than the initial bodies It is the opinion of the authors that the initial bodies are furnished with a capsular material of their own manufacture (as opposed to the matrix which appears to be derived from the cell) and that in the early stage both virus and capsule stain with methylene green or blue Later, due to metabolic changes, much of the virus retains the basic fuchsin while the capsule continues to stain green or blue This faculty of the virus to stain either with basic fuchsin or with methylene blue in Macchiavello's stain is shown by

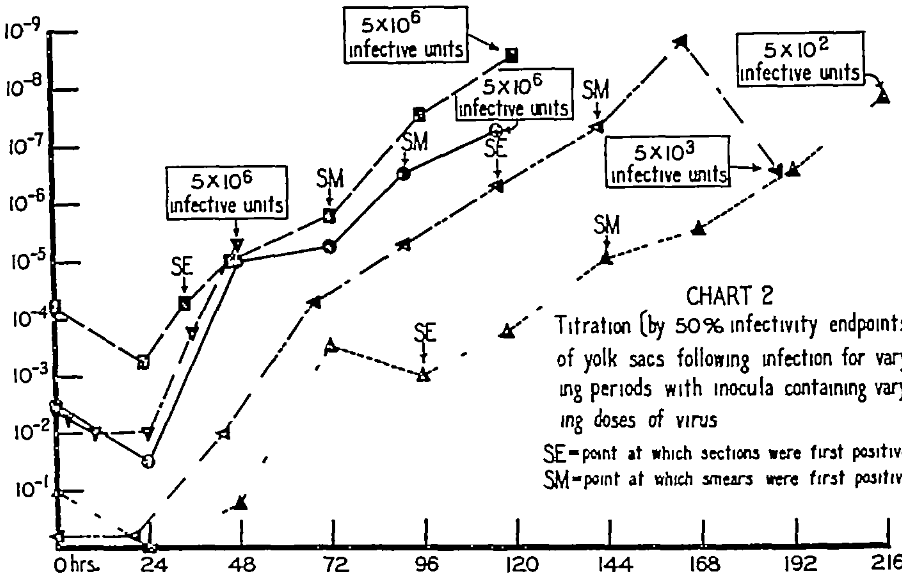

CHART 2
Titration (by 50% infectivity endpoints) of yolk sacs following infection for varying periods with inocula containing varying doses of virus
SE = point at which sections were first positive
SM = point at which smears were first positive

those bacteria that we have studied. The metabolic changes responsible are not understood The nature of either capsules or matrix has not been determined, it is not glycogen and in this respect the virus resembles that of psittacosis (32) and not that of trachoma (33)

When one correlates the changes in titre with the morphological appearances the following facts emerge During the "silent" period of 10 to 12 hours during which no virus is observed in the yolk cells, only a very slight rise in titre occurs Following the appearance of initial bodies, and coincident with the visual evidence of multiplication, the titre rises abruptly until about 16 or 18 hours Then, at the time when elementary bodies first appear in section and larger numbers of virus bodies appear in the smear, the titre drops abruptly and may take 9 to 12 hours to regain its former level It appears

probable that the appearance of elementary bodies is related to the rupture of many yolk cells and the discharge of virus into the yolk where most of it is lost in titrations of the yolk sacs This loss into the yolk obscures, for several hours, the continued increase within the yolk cells, but this period is finally succeeded by another and more prolonged one in which the titre again rises steeply

When smaller inocula are used as the initiating dose the results obtained are less spectacular and informative It is very difficult to follow the results as closely as can be done during the short period from inoculation to death which follows the giving of a large inoculum Two features which are usual in the titration curves obtained by the use of smaller inocula (Chart 2) merit attention The first occurs in the first 24 hours and is a drop in titre below that obtained immediately following inoculation It would appear to be due to a loss of infectivity on the part of many of the inoculated virus bodies The second, which occurs at varying times from 48 to 96 hours after inoculation, depending on the size of the original inoculum, is a break in the steep rise in the titration curve, or even a decrease in titre, seemingly corresponding to the break occurring in the curve following the large inocula (Chart 1) which has been shown to occur at the conclusion of a developmental cycle and the consequent liberation of elementary bodies into the yolk

DISCUSSION

Developmental cycles of equal or less complexity have been described in connection with other viruses. Thus Thygeson found larger initial bodies 0.3 to 0 8μ in diameter and smaller elementary bodies at a later stage in both inclusion blennorrhea (34) and trachoma (35, 36) As in lymphogranuloma venereum, division forms were frequent, in the vesicles or large colonies of elementary bodies the larger forms tended to lie at the periphery, and the larger bodies stained blue while the elementary bodies stained reddish blue with Giemsa. Thygeson noted the resemblance to the picture seen in psittacosis but pointed out (36) that the matrix surrounding the virus bodies in trachoma and inclusion blennorrhea could be shown to contain glycogen (33) which the matrix in psittacosis does not We ourselves have not been able to demonstrate glycogen in the matrix in lymphogranuloma venereum

Less resemblance to that of the agent of lymphogranuloma venereum is seen in the developmental cycle of vaccinia described by Bland and Robinow (37) but certain features of this latter cycle are reminiscent of the former

When however attention is turned to the life cycle of the virus of psittacosis it is found that the resemblance between this and that described above for lymphogranuloma venereum is very striking

Bedson and Bland were the first to draw attention to different developmental forms in psittacosis (32) Observing the spleens of infected mice they described, besides

the elementary bodies which stained deep purple with Giemsa, larger bodies of 1μ or more in diameter which might be circular or oval, appeared in pairs indicating division, and stained light blue with Giemsa In addition, moreover, there were homogeneous plaques several μ in diameter of material staining with Giemsa like the virus bodies, which apparently divided to give rise to the larger 1 μ bodies These in turn divided until the stage of elementary bodies was again reached, the whole cycle from elementary body back to the same stage taking 48 to 72 hours In later papers (38, 39) the sequence of events was studied more carefully It was found to be regular and completion of the cycle occupied 48 hours The infecting elementary bodies gave rise to larger 1μ bodies very soon after entering the cells These larger bodies multiplied as such and formed colonies embedded in a matrix Finally the large forms by subdivision gave rise to very large numbers of elementary bodies No conclusion was reached as to whether the elementary bodies multiplied as such When Bland and Canti (40) followed the actual process in tissue culture (of chick lung epithelium) they noted first, at 8 hours, round or oval 5 to 10μ plaques which increased in size Although these plaques appeared at first to be homogeneous, actually they contained many bodies 1μ in diameter Later still at 18 to 24 hours the matrix became less dense and forms intermediate in size between the 1μ bodies and elementary bodies could be seen and by 72 hours nothing but elementary bodies was seen, embedded in a very light matrix When these colonies of elementary bodies became enormous they eventually burst and set free numerous elementary bodies Like Gey and Bang with lymphogranuloma venereum (30) they noted that the elementary bodies of psittacosis which lay in a light matrix surrounded by a definite limiting membrane, exhibited violent brownian movement No development occurred outside the cells Levinthal (41) also described the developmental cycle in chick embryonic tissue culture He noted two types of cycle (*a*) as it occurs in normal cells with large forms and plaques exactly as described by Bedson and Bland, and (*b*) as it occurs in damaged cells in which case the elementary bodies as such divide to form enormous colonies (vesicles) of virus bodies Levinthal also noted what he termed involution forms, *i e* forms like dumbbells or others which were pale and vacuolated. Yanamura and Meyer (31) more recently have again investigated the developmental cycle in psittacosis and have confirmed in the main the results of the earlier investigators Particularly when one examines the illustrations given in the above papers on psittacosis is one struck by the marked similarity between the picture in this disease and that in lymphogranuloma venereum All the developmental forms described for the one disease can be exactly duplicated in the other

The finding of the close morphological similarity between the agents of lymphogranuloma venereum and psittacosis is of great interest in view of other resemblances which exist between these viruses Thus Rake, Eaton, and Shaffer (12) have shown that antigenic and tropistic similarities exist between the two viruses and others which appear to belong to the same group, and Jones, Rake, and McKee (11) have drawn attention to the fact that the carrier state may be as frequent and persistent in lymphogranuloma venereum as it is in psittacosis

Our own studies described above have confirmed most of the description of the cycle as given by Findlay and have filled in many details However, we have at no time seen any evidence to lead us to believe that the plaques are formed by aggregation of large bodies, rather one has been able to follow their formation by steadily increasing size of individual bodies, and the evidence for direct multiplication of elementary bodies as such would seem to be very strong since, for example, pairs and short chains are of common occurrence On the whole our interpretation of the different picture seen at different stages after infection resembles most closely that given by Levinthal (41) for the cycle of development of the virus of psittacosis In addition to the morphological changes we have been able to demonstrate constant changes in infective titre in the affected tissue and to correlate such changes to the morphological picture

Bedson (38) and Bedson and Bland (39) believed that the large bodies in psittacosis had less virulence for mice than had the elementary bodies and gave evidence in support of this claim. There is no reason to believe that in the case of lymphogranuloma venereum any such decreased virulence of large bodies for the embryo occurs and, in fact, a consideration of the facts points against it Thus, as is shown in Chart 1 or in Table II, an original inoculum of 5×10^8 infective units gives a titre of approximately $10^{-4.2}$ on immediate titration of the yolk sac. It is known that the infective units in this original inoculum are almost all elementary bodies They cannot be seen in the yolk cells in sections prepared immediately after inoculation nor is any virus seen *until about 10 hours when the larger initial bodies appear It seems more than* probable that such initial bodies, which are always single, when first seen and proceed shortly to divide, are derived by increase in size of the original elementary bodies. And this is borne out by the fact that the infective titre of the yolk sac remains approximately unchanged during this early period It certainly does not decrease as would be the case if these initial bodies had a virulence less than those of the elementary bodies from which they are derived

SUMMARY

Making use of the fact that the cells of the yolk sac of the developing embryo are readily infected with the agent of lymphogranuloma venereum and that the virus bodies can be readily observed in these cells because of the structure of the latter, the development of this agent has been followed at short intervals. It has been found to go through a regular cycle of development similar to that described for psittacosis in the spleen and less fully for lymphogranuloma venereum in the brain of infected mice The development as observed microscopically can be shown to run parallel to changes in the infective titre of the yolk sac as tested in other eggs

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EXPLANATION OF PLATES

Plate 9

Fig 1 Single virus body Eosin-methylene blue ×1200

Fig 2 Pair of virus bodies Eosin-methylene blue ×1200

Fig 3 Early vesicle containing several virus bodies Eosin-methylene blue ×1200

Fig 4 Larger virus bodies and groups Eosin-methylene blue ×1200

Fig 5 Larger virus bodies, one at top vacuolated. Eosin-methylene blue ×1200

Fig 6 Elementary bodies in plaque Noble ×1200

Fig 7 Group of plaques and large bodies Eosin-methylene blue ×1200

Fig 8 Multiple vacuolated plaques Eosin-methylene blue ×1200


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PLATE 10

FIG 9 Disintegrating and intact plaques Eosin-methylene blue × 1200

FIG 10 Vacuolated plaque Eosin-methylene blue × 1200

FIG 11 Disintegrating vacuolated plaque surrounded by elementary bodies Noble × 1200

FIG 12 Virus bodies in plaque Noble × 1200

FIG 13 Medium vesicle Eosin-methylene blue × 1200

FIG 14 Large vesicle Eosin-methylene blue × 1200

FIG 15 Three large vesicles Eosin-methylene blue × 1200

FIG 16 All cells filled with virus Eosin-methylene blue × 1200

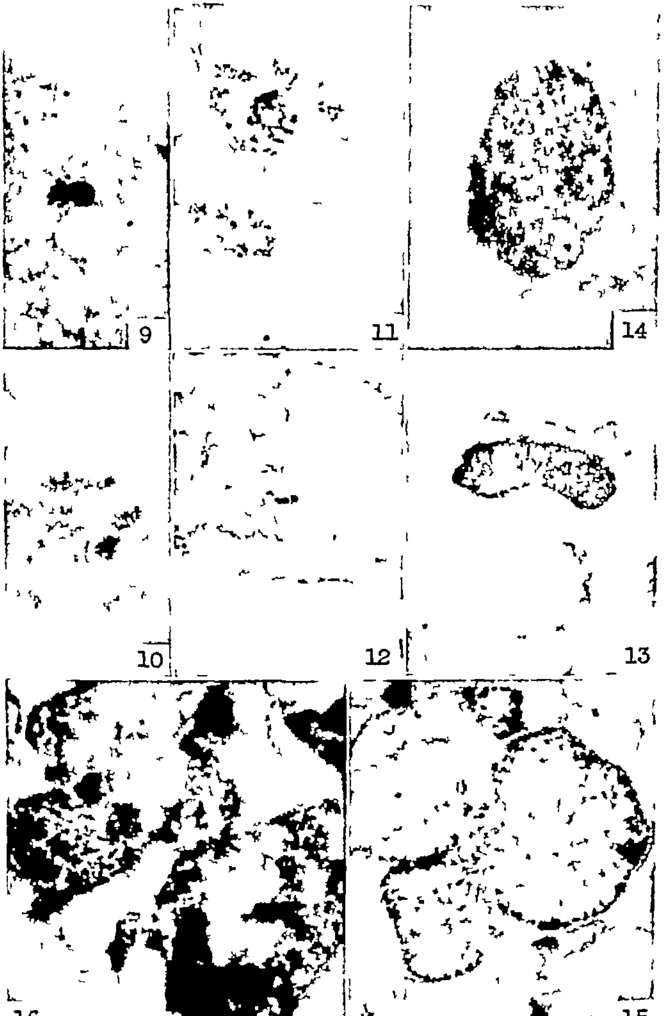
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Plate 11

Fig 17 Group of virus bodies in smear Giemsa × 1200

Fig 18 Large group of virus bodies in smear Giemsa × 1200

Fig 19 Group of elementary and larger virus bodies Smear Giemsa × 1200

Fig 20 Disintegrating plaque Smear Giemsa × 1200

Fig 21 Disintegrating matrix Smear Giemsa × 1200

Fig 22 Disintegrating matrix Smear Giemsa × 1200

Fig 23 Disintegrating matrix. Smear Macchiavello × 1200

Fig 24 Elementary and pair virus bodies Smear Giemsa × 1200

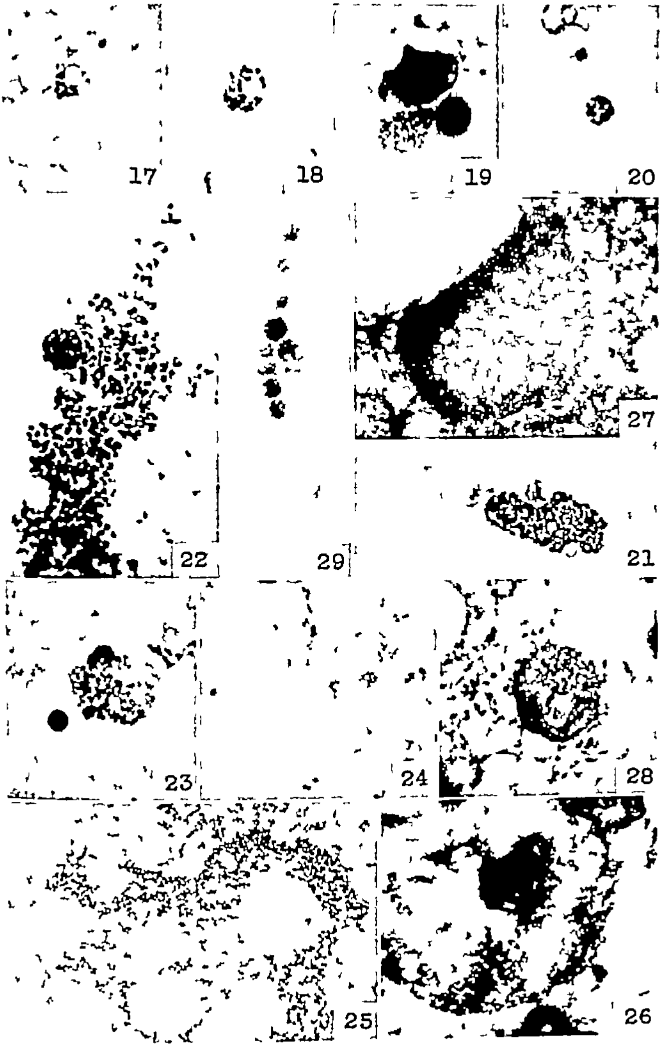
Fig 25 Elementary bodies Smear Giemsa × 1200

Fig 26 Elementary bodies in yolk cell Smear Giemsa × 1200

Fig 27 Large mass of virus from yolk cell Smear Giemsa × 1200

Fig 28 Phagocyted virus in monocyte Smear Giemsa × 1200

Fig 29 Colonies of staphylococci in yolk cells Eosin-methylene blue × 1200



Lymphogranuloma venereum

SEROLOGICALLY REACTIVE POLYSACCHARIDES PRODUCED THROUGH THE ACTION OF BACTERIAL ENZYMES*

I. Dextran of Leuconostoc mesenteroides from Sucrose

By EDWARD J HEHRE, M.D, and JOHN Y SUGG, Ph.D

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(Received for publication, December 16, 1941)

A preliminary paper (1) reported that a serologically reactive polysaccharide of dextran nature is produced from sucrose through the action of an enzyme or some similar heat labile agent contained in sterile filtered extracts of *Leuconostoc mesenteroides* The object of the present paper is to describe the preparation of the sterile extracts and to compare the chemical and serological properties of the dextran formed by the bacteria free extracts with those of the dextran formed in cultures of the living bacteria

Previous workers have reported the production of gum like material from sucrose by sterile filtrates derived from various species of spore forming bacilli. In 1910 Beijerinck (2) described the formation of "slime" on sucrose agar by filtered preparations of *Bacillus mesentericus*, he considered the active principle to be a "synthetically active enzyme" which he termed *viscosaccharase* Harrison, Tarr, and Hibbert (3) reported that a sterile filtrate of similar bacilli formed from sucrose an alcohol precipitable material which gave opalescent solutions. Also, it is possible that the production of bacteria free "colonies" on sucrose agar plates by filtrates of sucrose broth cultures of *Bacillus fluorescens* which has been reported by Dienes (4) and others represents another manifestation of the same phenomenon However, in none of the earlier studies were the products identified chemically or tested serologically, whereas in the present study the product is proved to be an immunologically reactive polysaccharide similar in both chemical and serological properties to the product formed in cultures of the living bacteria.

EXPERIMENTAL

Materials and Methods

Preparation of the Extracts—The extracts utilized in the present study were made from cultures of a strain of *Leuconostoc mesenteroides* isolated in this laboratory It gave mucoid growth on sucrose mediums and produced acid from both xylose and

* This investigation was aided by a grant from the Ruth B Ettinger Fund.

arabinose With this strain of leuconostoc the fundamental principle that serologically reactive material could be formed in the absence of the bacterial cells was demonstrable with Berkefeld filtrates of sucrose broth cultures That is, these filtrates which were apparently sterile as far as could be told by microscopic or cultural tests showed a gradual increase in opalescence and in serological reactivity when stored in the ice box. However, for a study of the formation of the polysaccharide the filtrates had the disadvantage of containing only a low concentration of the active agent in comparison to the large amounts of the preformed product That disadvantage was met by applying the principle introduced by Sevag (5) for the separation of proteins from polysaccharides when the fluids of sucrose broth cultures were shaken with chloroform most of the preformed polysaccharide remained in the supernatant, whereas the active principle which would subsequently produce the specific polysaccharide (when added to sucrose) was contained in the chloroform emulsion layer With this strain of leuconostoc (which has a highly soluble polysaccharide) the active agent could also be separated by salting-out procedures but the chloroform treatment was more convenient and was utilized to prepare all of the extracts used in the present experiments

Some extracts were made from the supernatant fluids of centrifuged cultures but for convenience most of them were prepared from the whole cultures without preliminary removal of the bacterial cells The usual starting material was a total of 5 one-liter lots of culture which had been grown in separate flasks for about 20 hours at 23°C , the cultures had received a large inoculum and by the time they were used had already become gummy or viscous, microscopic observation showed no evidence of autolysis or disintegration of the bacterial cells The medium in which the cultures were grown consisted of 1 per cent bacto-peptone (without meat extract or infusion), 0 5 per cent salt, and 5 per cent sucrose This medium afforded adequate growth and for the preparation of the extracts had the advantages of giving little foam when shaken and of containing only a minimum amount of material adsorbable upon chloroform

Each flask of culture was chilled, shaken by hand for 5 minutes with 25 cc of cold chloroform, and then centrifuged in the cold at 1500 R P M for 5 to 10 minutes The supernatant fluids which contained most of the serologically reactive polysaccharide and most of the bacteria were decanted from the chloroform emulsion precipitates which contained the active principle In order to get further separation from the preformed polysaccharide the emulsion material from all of the flasks was collected and washed two or three times with cold distilled water, each time the emulsion was suspended in 500 cc of water and then sedimented by low speed centrifugation and carefully separated from the wash fluid In order to free the active principle from its combination with the chloroform the washed emulsion material was treated with 500 cc. of cold 95 per cent ethyl alcohol, in which the active agent was insoluble The particles were brought into a finely dispersed state and this alcohol suspension was centrifuged in the cold at high speed for 40 minutes The alcohol was decanted and, after the tubes had drained for an hour in the refrigerator, the precipitate was extracted with 50 cc of distilled water and adjusted to about pH 7 5 with 0 1 N NaOH After removal of insoluble particles by centrifugation at high speed the extracts were filtered through Berkefeld W filters which had been

tested by the "bubbling pressure" method and found to emit air only at or above a pressure of 680 mm. of mercury The filtered extracts were adjusted to pH 6.0 and then stored in a brine-cooled chamber at a temperature slightly below 0°C.

Sterility Controls—Each extract was examined carefully by microscopic and cultural methods and in no instance were any microorganisms detected. No disinfectants were added to the extract-substrate mixtures but all of the constituents had been sterilized either by filtration or by autoclaving and aseptic technique was employed in the preparation and subsequent handling The sterility of the extract substrate mixtures was well controlled by microscopic and cultural tests made at appropriate intervals during the incubation periods as well as at the beginning and end of the experiments The cultural tests consisted of inoculations in sucrose peptone broth and agar slants and also in broth and agar slants enriched with sterile (filtered) unheated cane juice all these mediums were known to be adequate for growth of leuconostoc bacteria and those enriched with the unheated cane juice can be regarded as furnishing an especially rigorous test. From the results of the numerous sets of controls we feel certain that all of the observed reactions occurred in the complete absence of bacteria.[1]

Potency of the Extracts—The present paper does not include any systematic data on the minimal time and the minimal concentration of extract which are required for the production of demonstrable amounts of the polysaccharide, but some information on both those points was obtained. In respect to time, if the extract was used in dilutions of 1 2 or 1 4 in mixtures containing 5 per cent sucrose significant amounts of reactive material were regularly produced within 1 to 2 hours at either 23 or 37°C In respect to minimal amount of extract, dilutions of 1 1000 of all and of 1 10,000 of most of them would form demonstrable amounts of the polysaccharide in test mixtures containing 5 per cent sucrose if incubated for 20 days at 23°C

Serological Tests—All of the antiserums were from rabbits The dextran of the present strain of leuconostoc reacts with Types 2 and 20 antipneumococcus as well as with antileuconostoc serum (6, 7) in the many comparative titrations which we have made the range of dilutions of the dextrans which gave precipitation was usually essentially the same for those three kinds of antiserum. Hence, in most experiments of this paper the dextran produced by action of the extracts was measured on the basis of tests with only one of the antiserums, usually Type 2 antipneumococcus. The dextran also reacts to some extent with Type 12 antipneu

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[1] Tubes of sucrose broth if inoculated with as much as 0 5 cc. of the extracts became opalescent but the opalescence can be accepted simply as an intrinsic property of the polysaccharide product formed from sucrose by the active principle of the extracts, no bacteria were demonstrable in the opalescent solutions either by direct examination or by subculture, and no change in pH or titratable acidity was evident whereas with leuconostoc bacteria an increase in acidity would have occurred. Further evidence that the opalescence did not represent microbial growth was that its development was neither more rapid nor finally greater in amount when the extracts were inoculated in sucrose mediums (broth or unheated sugar cane juice) which could support luxuriant growth of the bacteria than when the same extracts were added to aqueous sucrose solutions in which leuconostoc bacteria could grow poorly if at all.

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mococcus serum but since this cross reaction occurs only with relatively high concentrations of the antigen the Type 12 antiserum by itself was unsuitable for routine measurement of the product of the action of the extract

The leuconostoc antiserums were produced by immunization with bacteria grown in sucrose peptone broth, none of the antiserums we have obtained with leuconostoc grown on glucose mediums have been reactive with the dextran The pneumococcus antiserums had been produced by immunization with bacteria grown in infusion broth containing no added carbohydrate, with the pneumococci growth in a sucrose medium apparently is not essential in order to get an antiserum reactive with the leuconostoc dextran It should be noted that an occasional lot of either Type 2 or 20 antiserum although of high homologous titre may lack the capacity to give the usual leuconostoc reaction (6)

Action on Sucrose

A number of lots of sterile extract prepared at different times by the described procedure have been tested and all have had the capacity of forming serologically reactive material from sucrose The extracts obviously do not represent purified solutions of the active agent but were adequate to establish the general principle of the reaction with sucrose and to permit the isolation of the purified polysaccharide product in amounts large enough for chemical as well as serological study None of the extracts was entirely free of preformed polysaccharide However, the amount contained was extremely small in comparison to the yield obtained after incubation with sucrose, so that there could be no doubt that by far the principal part of the polysaccharide found in the incubated test mixtures represented material synthesized through the action of the sterile extract

The reaction with sucrose was recognizable not only by serological test but also by simple observation of the development of opalescence and by chemical tests for alcohol-precipitable material and for reducing sugars The set of phenomena which always characterized the action of the sterile extract is illustrated by the data of the following experiment

A series of mixtures consisting of one part of sterile extract plus one part of sterile (filtered) 10 per cent sucrose[2] solution in 0 2 molar acetate buffer pH 5 6 was prepared in a series of tubes, for controls similar mixtures with heat-inactivated (30

[2] The sucrose used throughout the investigation was a sample of beet sugar known by previous test to be free from material reactive with the antiserums (leuconostoc and Types 2, 20, and 12 pneumococci) with which the leuconostoc dextrans are reactive It was used instead of reagent sucrose because the latter usually contains significant amounts of material reactive with leuconostoc or the related pneumococcus antiserums (9, 10) The use for substrate of sucrose free of polysaccharide which is either identical or closely related to the polysaccharide product of the action of the extract seemed important for theoretical reasons, particularly because of the possible stimulatory influence of the presence of small amounts of the final product

minutes at 55°C.) extract were included the incubation was at 23°C At the time intervals listed in Table I the mixtures were observed for opalescence and one tube of each series was used for tests for alcohol precipitable material serological reactivity, and reducing sugar (8) The alcohol precipitated material was sedimented by centrifugation and redissolved in salt solution equivalent to the original volume of the sample, this solution was observed for opalescence, tested for serological reactivity and for amount of reducing sugar before and after acid hydrolysis for the hydrolysis the solution was heated with 1 0 N sulfuric acid for 6 hours in a sealed tube immersed in boiling water The serological reactivity was determined by test against 1 15 dilutions of a Type 2 antipneumococcus rabbit serum the precipitation reaction was observed after 1½ hours incubation at 37°C In the summary of the results presented in Table I the reducing sugars are calculated as glucose.

TABLE I

Action of the Sterile Extract upon Sucrose

Test mixture	Incubation period	Properties of test mixtures				Properties of solutions of the alcohol precipitate		
		Opalescence	Precipitability with 1.5 volumes of alcohol*	Serological reactivity‡	Reducing sugars (without hydrolysis)	Opalescence	Serological reactivity‡	Reducing sugars after acid hydrolysis§
	hrs.				*mg./cc.*			*mg./cc.*
Unheated extract plus sucrose	0	0	±	10	0 07	0	10	0 04
	6	+	++	200	0 4	+	200	0 3
	12	+±	+++	400	1 0	++	400	0 9
	24	++	++++	1000	2 4	++	1000	2 0
	48	+++	++++	1500	4 0	+++	1500	3 9
	96	++++	++++	3000	7 8	++++	4000	6 6
	192	++++	++++	7000	10 8	++++	7000	9 0
Heated extract plus sucrose	96	0	±	10	0 08	0	10	0 02
	192	0	±	10	0 09	0	10	0 02

* Test mixture diluted 1 5 in 10 per cent sodium acetate before addition of the alcohol.

‡ Dilution which gave precipitation with Type 2 antipneumococcus serum.

§ Only traces (0 02 to 0 03 mg per 1 cc.) of reducing sugars before hydrolysis.

It is evident (Table I) that the development of the different phenomena (opalescence, serological reactivity, material precipitable with 1.5 volumes of

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of the enzymatic action. From the practical standpoint of possible confusion in the final serological tests the presence of reactive material in the sucrose substrate would be important only in the serological test of relatively low dilutions of enzyme-substrate mixtures Since all the experiments reported were done with sucrose derived from beet it should be noted that a sufficient number of well controlled experiments were made with cane sugar as substrate to prove that the sterile extracts can also form the reactive polysaccharide from sucrose of that origin.

alcohol, and free reducing sugar) proceeded at an orderly rate when the unheated extract was incubated with sucrose, and that none of those phenomena occurred in the control mixture of sucrose plus heated extract

The alcohol-precipitable material can be accepted as the product responsible for both the opalescence and the serological reactivity of the test mixture, the free reducing sugar represents another product of the action of the bacterial extract upon sucrose It is important that throughout the course of the reaction the amount of free reducing sugar which had accumulated in the test mixtures was about the same as the amount of reducing sugar obtained by acid hydrolysis of the alcohol-precipitated material This approximate agreement suggests that the action of the extract consists of the conversion of x molecules of sucrose into a polymer of x glucose anhydride units plus x molecules of fructose Although that mechanism is not proved, data to be presented in Table II show that the polysaccharide is a dextran and other experiments which we have made indicate that the free reducing sugar is fructose [3]

Chemical and Serological Properties of the Purified Polysaccharide Product

The facts (Table I) that the serological reactivity which developed in the sucrose-extract mixtures was resident in the alcohol-precipitable fraction and

[3] The supernatant fluids of extract-sucrose mixtures from which the dextran had been removed by alcohol precipitation, yielded large amounts of glucosazone (identified by crystalline appearance and by melting point) when heated with phenylhydrazine and sodium acetate That the material from which the glucosazone was derived was a product of the action of the extract upon sucrose was proved by the lack of formation of glucosazone when the supernatant fluids of mixtures of heat-inactivated extract plus sucrose were subjected to similar treatment These results indicated that the reducing substance was either glucose, mannose, or fructose

More specific evidence was obtained by experiments utilizing the procedure of Ekkert (11) (the low concentration of the reducing substance in our test mixtures prevented the use of the methylphenylhydrazine method and the presence of sucrose in the mixtures prevented the use of the usual color tests for fructose) The method consists simply of the addition of two or three drops of 4 N NaOH and a small piece of solid NaOH to two or three drops of the test solution on a porcelain plate if fructose is present a pink to blood red color develops which is not given by other common carbohydrates When this procedure was applied to mixtures of sucrose plus active extract which contained from 0 3 to 1 0 per cent of free reducing sugar, they always gave the pink to red color Control tests with 0 3 to 1 0 per cent solutions of reagent fructose gave pink to red colors of intensities comparable to those shown by the sucrose-extract mixtures of corresponding reducing sugar content Similar tests made upon mixtures of heat-inactivated extract plus sucrose, upon solutions containing 1 and 10 per cent sucrose, glucose, galactose, xylose, arabinose, maltose, or lactose, gave either a yellow color or no color, the purified leuconostoc dextran (1 0 per cent) gave no color Thus, although the reducing sugar formed by the action of the extract upon sucrose was not actually isolated and identified there is considerable reason to believe that it is fructose

that this fraction yielded abundant amounts of reducing sugar upon acid hydrolysis indicated that the product responsible for the serological reactivity was a polysaccharide It seemed important to obtain purified preparations of this product in sufficient amount for an adequate comparison of its chemical and serological properties with those of the dextran polysaccharide which is formed in sucrose broth cultures of the leuconostoc bacteria from which the extracts had been derived A preparation of dextran from sucrose broth cultures was available from another investigation (6) and for the present experiments two lots of polysaccharide were prepared utilizing sterile extracts made at times several months apart.

For lot 1 the test mixture was 20 cc. of bacterial extract plus 380 cc. of sterile 10 per cent sucrose in 0 1 M acetate buffer solution pH 5 6 for lot 2 the mixture was 300 cc. of the buffered sucrose solution plus 15 cc. of the bacterial extract the first lot was incubated 9 days and the second lot 7 days at 23°C. Control mixtures (heat inactivated extract plus sucrose and unheated extract plus buffer) were also prepared and incubated for the same periods. At the end of the incubation period the test mixtures were opalescent and apparently contained adequate amounts of the polysaccharide product the control mixtures remained clear and furthermore gave no precipitate upon addition of 10 per cent sodium acetate and 1 5 volumes of alcohol.

The polysaccharide material from lots 1 and 2 was isolated and purified by the procedure described for preparation of the dextran from broth cultures (6) After addition of 10 per cent sodium acetate, 1 5 volumes of alcohol were added to the extract-substrate mixtures the abundant gummy precipitate was dissolved in a volume of water equal to that of the original mixture and was then reprecipitated in the presence of acetate by the addition of 1.25 volumes of alcohol This precipitate was redissolved in 300 cc. of water and after the addition of 10 gm. of sodium acetate and 5 cc. of glacial acetic acid was shaken mechanically with chloroform to remove any traces of protein (5 12) The chloroform-protein emulsion layer was only small in amount after the first treatment and was entirely absent after the third treatment. The protein-free material was then twice precipitated by 1.25 volumes of alcohol in the presence of acetate the final precipitate was ground under absolute alcohol and dried in vacuum over $CaCl_2$ at room temperature. The yield was 0 70 gm of the purified polysaccharide from lot 1 and 0 45 gm. from lot 2, which represents, respectively, 175 and 143 mg per 100 cc. of the extract-sucrose mixtures. It is noteworthy that the yields obtained through the action of these sterile extracts is greater than the yields of polysaccharide ordinarily obtained from whole cultures of pneumococci and many other bacteria.

Both of the purified preparations were analyzed chemically and tested serologically The specific optical rotation (sodium D line at 24°C.) of the original products was determined with 0 4 per cent solutions For determining the properties after hydrolysis 0.2 gm. of each product was heated for 6 hours with 1 0 N sulfuric acid in a sealed tube immersed in boiling water in addition to the analyses for reducing sugars (8) and for optical rotation, the hydrolysate was treated with phenylhydrazine and oxidized with nitric acid (13) *in order to get more specific information on the nature* of the reducing sugar in the hydrolyzed material.

periments It was known from a previous study (6) that absorption with leuconostoc bacteria would remove the capacity to react with the dextran of culture source from all of the antiserums listed in Table III provided the leuconostoc used for the absorption had been grown in sucrose broth, whereas absorption with pneumococci (2, 20, or 12) would fail to remove the dextran-reacting capacity from any of the antiserums other than the homologous antipneumococcus type When these experiments were repeated with dextran of extract origin together with dextran of culture origin, the two behaved exactly alike, that is, the absorption treatment which removed the antibodies reactive with one always removed the antibodies reactive with the other, and the treatments which failed to remove the antibodies reactive with one always failed to remove the antibodies reactive with the other [5]

In spite of the striking similarity in the serological properties from a qualitative standpoint, it should be noted that one lot (No 1) of dextran of extract source did not react in as high dilution as did the dextran isolated from the culture However, this difference seems relatively unimportant because both lots (Nos 1 and 2) of extract origin reacted in sufficiently high dilution (1 500,000 and 1 1,000,000) to prove the fundamental point that polysaccharides produced in the absence of living cells can approach if not equal the quantitative serological capacity of the polysaccharides produced in the usual sucrose broth cultures of the bacteria

Influence of pH upon the Action of the Extract

The substrate-extract test mixtures in all the previously described experiments were maintained at about pH 5 6 by the use of acetate buffer That routine was adopted at the beginning of the investigation because of an early observation that the extracts acted upon sucrose more rapidly at that pH than at pH 7 0 In order to get more systematic information on the influence of pH the following experiment was made

A series of mixtures consisting of one part of extract, one part of 20 per cent sucrose, and two parts buffer solution were prepared, which gave test systems ranging from

[5] In addition to furnishing evidence on the likeness of the dextrans from the two sources, these experiments illustrate an interesting difference between Type 2 pneumococci and leuconostoc bacteria in respect to the influence of growth in sucrose broth upon the antigenic properties of the two sorts of bacteria (6, 15) That is, the capacity to evoke antibodies reactive with the leuconostoc dextran or to remove the anti-dextran antibodies from a reactive serum, is possessed by leuconostoc bacteria which have been grown in sucrose broth but not by leuconostoc bacteria which have been grown in glucose broth, whereas Type 2 pneumococci which have been grown in plain or in glucose broth can not only evoke the dextran antibodies but also can absorb them from antiserums produced by immunization with Type 2 pneumococci which have been grown either in sucrose or non-sucrose broth

pH 3 0–9 0, citrate buffer was used for the pH 3 0, acetate for the pH 4.0, 5 0, and 5.5, phosphate for the pH 6 0, 7 0, and 8 0, and borate for the pH 9 0 mixture. The pH which was tested at the beginning and again at the times of each serological determination, remained approximately constant in all the mixtures except the pH 9 0 borate which dropped to pH 8 5 early in the experiment The test mixtures were incubated at 23°C. In order to measure the formation of serologically reactive material samples were removed from each mixture at the times shown in Fig 1 and tested in a series of dilutions against a 1 15 dilution of Type 2 antipneumococcus serum the samples were adjusted to about pH 7 0 by diluting them 1 10 in 0 1 M phosphate solution before using them in the serological tests.

It is evident (Fig 1) that the extract formed significant amounts of the serologically reactive material over the relatively wide zone of pH 4 0–8 0 but that

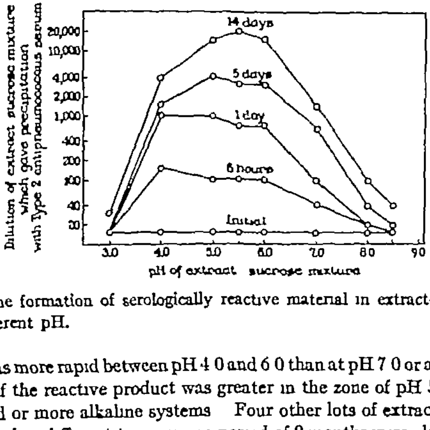

FIG. 1 The formation of serologically reactive material in extract-sucrose mixtures of different pH.

its action was more rapid between pH 4 0 and 6 0 than at pH 7 0 or above The final yield of the reactive product was greater in the zone of pH 5 0–6 0 than in more acid or more alkaline systems Four other lots of extract which had been prepared at different times over a period of 9 months were also compared in test systems of pH 5.5 and 8 2 All of these extracts produced at least 100 times more reactive material after 5 days incubation at 23°C in the pH 5.5 mixture than in the pH 8.2 mixture, which indicates that the results in Fig 1 are representative of extracts prepared from this strain of leuconostoc.

Influence of Temperature of Incubation

The routine temperature of incubation of the substrate-extract test mixtures was 23°C The enzyme, however, had the capacity of forming the serologically reactive material over a range of at least 3–37°C In experiments made with 5 different lots of extract, evidence was obtained that considerable

inactivation of the enzyme occurs in mixtures incubated at 37°C For example, the amount of serologically reactive material formed at 37°C was about the same as that formed at 23°C during the first 5 hours of incubation, but after periods of 3 days the mixtures incubated at 23°C invariably contained at least 5 times as much of the reactive product as did the corresponding mixtures incubated at 37°C This apparent slowing of the reaction at 37°C became even more apparent after 10 and 20 days by which time the mixtures incubated at the low temperature of 3°C contained about 5 times as much of the reactive product as that in the mixtures incubated at 37°C The amount formed at 3°C never equalled that produced by the same extract at 23°C during the 20 day period of the experiment However, the fact that 3°C was sufficient for a reasonably high degree of activity on the part of the leuconostoc enzyme is of interest because at that temperature cultures of those bacteria will grow little if at all

Lability of the Active Agent

The active agent of the extracts is heat-labile control mixtures of heated extract (30 minutes at 55°C) plus sucrose were included in each experiment throughout the investigation and in no instance was any reactive material formed This routine heating treatment was evidently more than sufficient since 5 minutes exposure to either 50° or 55°C usually resulted in practically complete inactivation whether the extracts were heated in systems of pH 5 5 or pH 7 0

Observations on the question of lability at 37°C were made with several lots of extract The active agent in all of them was destroyed more rapidly when the exposure (in the absence of sucrose) was in systems of pH 7 0 than when in systems of pH 5 5 Individual extracts varied in the degree to which they showed this difference With some the difference was great for example, one extract lost at least 90 per cent of its original activity when it was exposed for 4 hours at pH 7 0 whereas only a slight loss in activity occurred when it was exposed for the same time at pH 5 5 Controls were included to show that the greater lability at 37°C on the part of extracts held at pH 7 0 was not due to any action of the phosphate which was used as buffer The difference in the degree of lability at 37°C which occurred among the individual extracts suggests that some unrecognized impurity present in different concentrations in the various extracts may have participated in the destruction of the active principle of the extract Experiments will be made to determine whether or not the inactivation at this comparatively low temperature represents an oxidative process

Active Extract from a Glucose Broth Culture

Cultures of *Leuconostoc mesenteroides* produce abundant amounts of the reactive dextran when grown in sucrose medium but produce none of it when

grown in mediums in which glucose or other common carbohydrates are substituted for sucrose The failure of the bacteria to produce the dextran in the latter mediums can reasonably be explained on the basis that these mediums lack the substrate (sucrose) required for the synthesis of the dextran Nevertheless, it seemed of interest to determine whether or not the bacteria after repeated passage in a medium lacking sucrose would elaborate the enzyme involved in the dextran synthesis. For the investigation of that question the leuconostoc bacteria (which previously had always been grown in sucrose mediums) were transferred 9 successive times in glucose broth, and an extract was then prepared by exactly the same procedure as previously described

This extract was found to possess the capacity to form the polysaccharide when added to sucrose, but its potency was low for example, the extract derived from the glucose culture when used in a dilution of 1 2 produced about the same amount of polysaccharide as did 1 50 or 1 100 dilutions of the extracts derived from sucrose cultures. These results show that the leuconostoc bacteria can elaborate some amount of the enzyme involved in the dextran synthesis when grown in the absence of sucrose However, the lower degree of reactivity of the extract derived from the glucose culture indicates that the presence of sucrose during the growth of the bacteria has a stimulatory influence upon the elaboration of the enzyme.

DISCUSSION

The experiments dealt with the production from sucrose of a serologically reactive polysaccharide of dextran nature by the action of sterile filtered extracts obtained from cultures of *Leuconostoc mesenteroides* That other common carbohydrates do not yield the polysaccharide which is formed from sucrose was shown previously (1) and further information on that point will be given later The active principle in the extracts was heat labile and presumably was an enzyme or combination of enzymes. From the method of preparation it can be assumed that the active principle was "exocellular" or free in the fluid of the cultures from which the extracts were obtained. However, although it was a less convenient method, active extracts were also obtained by grinding the bacterial cells which indicates that some of the active agent also occurs within or upon the surface of the cells

In regard to chemical properties the polysaccharide formed by the sterile extract was similar to the polysaccharide produced in sucrose broth cultures, both could be classified as dextrans on the basis of their high positive optical rotation high content of reducing sugars after hydrolysis and by evidence that glucose comprised at least the principal portion of the hydrolyzed products In regard to serological properties the dextrans of extract and of culture source were strikingly similar not only in tests against leuconostoc antiserum but also in tests against Types 2, 20, and 12 antipneumococcus serums and in tests against a series of variously absorbed antiserums. This agreement in the

antipneumococcus cross reactions and in the reactions against the absorbed serums represents evidence of a closer serological likeness between the dextrans of the two sources than would be furnished by tests against leuconostoc antiserum alone

No intensive study of the mechanism of the action of the sterile extracts was made other than to show that the products are formed at a reasonably orderly rate and that the reaction, which occurs to some extent over the relatively wide zone of pH 4 0–8 0, is more rapid and gives a higher final yield in systems between pH 5 0 and 6 0 than at pH 7 0 or above It is of interest to point out that the elaboration of the dextran by the active agent of leuconostoc origin has at least two points in common with the syntheses of glycogen and starch by enzymes of yeast, animal, and plant origins That is, in regard to the final product of the reaction, the leuconostoc dextran like glycogen and starch is a complex polysaccharide composed apparently entirely of glucose units, and in regard to the substrate, the sucrose from which the dextran is derived, like the glucose-1-phosphate from which glycogen and starch are formed, is a glucoside But the mechanism of formation of the dextran has not been studied enough to allow any closer comparison, for example the questions of whether or not phosphorus compounds participate and of whether or not the reaction is reversible have not been determined

Although the experiments of this paper included extracts prepared from only one strain of *Leuconostoc mesenteroides*, the possession of a demonstrable polysaccharide-synthesizing enzyme is not peculiar to this strain Sterile extracts which form serologically reactive polysaccharides similar to those produced in the corresponding bacterial cultures, have been prepared not only from 5 other strains of leuconostoc but also from group H streptococci, *Streptococcus salivarius*, and several non-spore-forming bacilli (presumably lactobacilli) isolated from plants However, for the demonstration of the "enzymatic synthesis" of serologically reactive polysaccharides, all of these kinds of bacteria seem to have at least two advantages over the majority of microorganisms First, the extraordinarily large amounts of the polysaccharide found in the whole cultures would indicate them to be a likely source from which to isolate a sufficient amount of enzyme or active agent to produce demonstrable amounts of the polysaccharide in the absence of the cells Secondly, the formation of the polysaccharide by these bacteria can be referred to a definite constituent (sucrose or raffinose) of the medium Of these two advantages the knowledge of the substrate is probably the more important, and indeed the other apparent advantage may depend entirely upon it For example, in the case of leuconostoc and of the other kinds of bacteria from which we have prepared active extracts, the extraordinarily large amounts of polysaccharide are produced only when the medium contains relatively large amounts of the particular substance (sucrose and sometimes raffinose) from which the polysaccharide is elaborated, if none of that substance is available none of the polysaccharide

is produced In the case of pneumococci and most other bacteria the actual substrates from which the specific polysaccharides are made are entirely unknown not only in regard to their chemical nature but also in regard to whether they represent original constituents of the medium or some intermediate products of bacterial metabolism But in either event if it were possible to supply the appropriate substances in adequate amount the yield of specific polysaccharide might perhaps become as abundant as the yield of dextran in sucrose broth cultures of leuconostoc.

SUMMARY

A serologically reactive polysaccharide of dextran nature was produced from sucrose by the action of some enzyme or similar heat labile agent contained in sterile filtered extracts derived from sucrose broth cultures of *Leuconostoc mesenteroides* Rigorous controls were included to prove that this reaction occurred in the absence of microorganisms.

Purified preparations of the dextran formed by the sterile extracts were similar to the dextran elaborated in sucrose broth cultures of the bacteria in respect to both chemical and serological properties. The serological likeness was established not only by tests against leuconostoc antiserums but also by cross reactions with antiserums of Types 2, 20, and 12 pneumococci and by tests against a series of variously absorbed antiserums.

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OBSERVATIONS ON THE CONDITIONS OF DIETARY HEPATIC INJURY (NECROSIS, CIRRHOSIS) IN RATS*

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PLATE 12

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It was reported in 1939 (2) that hepatic injury, mainly in the form of acute focal or diffuse necrosis combined with fat infiltration, occurred irregularly in young rats fed a diet devoid of vitamin B (casein 18 per cent, cane sugar 68, melted butter fat 8, cod liver oil 2, salt mixture 4) and supplemented with thiamine, riboflavin, and pyridoxine. In a group of more than 300 rats, 48 exhibited hepatic changes of the type described. In livers of 4 of the rats there was diffuse periportal fibrosis.

Admittedly, even when hepatic injury was observed under apparently identical experimental conditions, it was found that it "could not be produced at will and was not a regular occurrence" No proof for an infectious or toxic origin of the pathological changes in the liver could be found and the evidence favored, rather, a nutritional basis (2) In this connection it is particularly noteworthy that injury to the liver was never encountered in rats which, for from 3 to 6 months, had been fed a diet deficient in the vitamin B complex and supplemented with thiamine, riboflavin, and 0.5 gm. of yeast daily Nor was hepatic injury found in investigations on riboflavin deficiency (3, 4) involving approximately 500 rats which were fed the same basal diet supplemented with thiamine and yeast extract in the form of Peters' eluate (5) As this hepatic disease was not prevented by the administration of thiamine, riboflavin, and pyridoxine, it was assumed (2) that the hypothetical factor which protected the liver must be one that was different from these components of the vitamin B complex.

The incidence of hepatic injury remained practically unchanged in later groups of at least 250 rats[1] fed a vitamin B free diet, with daily supplements of 20 micrograms of thiamine, 20 to 25 micrograms of riboflavin, and 2 mg of choline per gm. of diet (or 10 mg of choline daily as a separate supplement)

* A preliminary report of this work has been made (1)

[1] In this number is included the series of 169 rats referred to in footnote 1 of an earlier report (6)

and with or without the further addition of pyridoxine Injury to the liver was also observed in rats which had received pantothenic acid because manifestations of pantothenic acid deficiency had become apparent on a diet supplemented with the factors mentioned If the rats treated with pantothenic acid, however, survived the first 4 weeks of medication with distinct gain in weight, they remained permanently free from hepatic injury

Another group of 25 young rats, weighing between 30 and 35 gm , which had received from the start all the supplements in question (vitamin B_1, riboflavin, pyridoxine, pantothenic acid, and choline), exhibited no specific hepatic changes even for as long as 8 months after the beginning of the experiment

One common denominator emerged as a conceivable etiologic factor out of the multitude of these variable observations This was the possibility of low food intake with, at the same time, a sufficient supply of several or all the known members of the vitamin B complex Limitation of hepatic injury to rats suffering from or convalescing from deficiency of one or more members of the vitamin B complex is consistent with this assumption, as the intake of food is probably especially low and variable under these conditions On the other hand, the fact that hepatic injury is lacking in rats whose fairly normal development is not interrupted by deficiency conditions also tallies well with this view As a further logical step it could be assumed that the low food intake is associated with low casein supply and therefore, in natural consequence, with the so called lipotropic activity of casein

As the amounts of food ingested were not determined, special investigations were needed in order to make such a tentative conclusion more plausible In the experiments which are reported here, an attempt has been made to employ different rations that were low in protein and moderately or distinctly high in fat.

In the meantime Rich and Hamilton (7) have reported the occurrence of cirrhosis of the liver in rabbits maintained on a deficient diet and its prevention by yeast The exact nature of the deficiency was not determined. Spellberg and Keeton (8) also observed cirrhosis of obscure dietary origin in one guinea pig and one rabbit In this connection it should be borne in mind that herbivorous animals, especially rabbits, are not altogether satisfactory experimental subjects for the study of hepatic cirrhosis "Reagents which produce the disease in them fail to have the same effect on dogs, and from the spontaneous cirrhosis which occurs they would seem to be peculiarly susceptible to the disease" (9)

The production of cirrhosis of the liver in 3 dogs and in 7 rats fed high fat diets has been reported by Chaikoff and Connor (10) and by Blumberg (11), respectively These results, however, were correlated not to the phenomenon of the lipotropic activity of casein but rather to the fat infiltration of the liver

Experimental Method

Rats with an initial weight of 130 gm. or more, in groups of 10 or more, were fed seven different rations, the composition of which is summarized in Table I In all

but a few groups the diets were supplemented, as a matter of daily routine, with 20 micrograms of thiamine 25 micrograms of riboflavin, 20 micrograms of pyridoxine, and 100 micrograms of calcium pantothenate.[2] The daily intake of food was not determined. In a few groups 0.5 gm of dried brewer's yeast (Anheuser Busch, Inc.) was substituted for the vitamin supplements, three times a week. The vitamin solutions and the yeast, as well as the other special supplements, were given separately, that is, they were not mixed with the diet

The feeding period of all the different groups was extended to 150 days or, exceptionally, a few days longer Pathological examination of rats that died before the termination of the experiments followed as soon after death as possible.

In the overwhelming majority of the animals the diagnosis was made only after macroscopic and microscopic examination of the liver As stated in the previous report (2) in a few rats the excited, hyperactive behavior might hint the presence of disease of the liver Visible jaundice (yellow ears, bile stained urine) was observed

TABLE I

Composition of the Diets

Constituent	Diet S I	Diet L II	Diet L III	Diet L IV	Diet L V	Diet C I
Casein smaco	18	10	6	18	5	8
Lard		20	23	20	20	
Crisco						38
Melted butter fat	8					
Cane sugar	68	64	15	56	69	48
Corn starch			50			
Cod liver oil	2	2	2	2	2	2
Salt mixture (12)	4	4	4	4	4	4

even less frequently In the rats in which cirrhosis was found at autopsy, greasy sparse, matted fur, and brown seborrheic adherent scales on the skin chiefly over the back were often observed in life. Clinical ascites was suspected in only one rat of all the groups under investigation. At autopsy, however ascites alone and in the presence of especially advanced cirrhosis, ascites with pericardial and pleural effusion were observed several times although still rarely The effusion fluid was found to be frequently bloody Hematemesis and consequent anemia were equally exceptional findings. In these rats the anemia was hypochromic for instance, in rat 6473 the blood count was hemoglobin 45 per cent, red blood cells 3,990 000, white blood cells 6,900, polymorphonuclear cells 11 per cent, small mononuclear cells 85 per cent, large mononuclears 4 per cent.

The growth curve of rats suffering from hepatic injury (necrosis or cirrhosis) exhibited varying tendencies, although as a rule, at least in the last stages of the disease,

[2] Thiamine chloride riboflavin, pyridoxine, and calcium pantothenate were obtained from Merck & Co , Inc , Rahway, New Jersey purified casein, choline chloride, *l*-cystine, and tyrosine from the S M A. Corporation Chagrin Falls, Ohio and *dl*-methionine from both of these sources

retardation and even suppression with final loss of weight seemed to prevail. In many animals, however, milder forms of cirrhosis were not incompatible with progressive gain in weight.

The investigations reported here have been carried out during the last 3 years on over 360[3] rats. These experiments are being continued with special emphasis placed on the problem whether or not cirrhosis of the liver once produced can be influenced therapeutically. Thus far the special dietary factors have been tested only for the purpose of determining interference with the development of the lesions.

Pathological Findings

In the livers of the rats in the various groups that received different percentages of protein and different supplements there is a great variety of pathological changes. Fat infiltration in some degree is almost invariably present. Less frequent is parenchymatous or fatty degeneration with variable degrees of fat infiltration. The most significant changes are diffuse or focal necrosis, with or without accompanying hemorrhage, and variable degrees of cirrhosis. As a rule, the necrosis is recognizable in the gross but a considerable degree of cirrhosis may exist without being obvious in the gross. The necrosis is mainly central and midzonal, but frequently a variable number of entire or almost entire lobules is the seat of this change. In some livers large portions of a lobe are necrotic, but thrombosis, which might also account for such massive necrosis, was not observed. In Tables II to IV, under the heading "necrosis" are included those livers which showed only this change. Under the heading "cirrhosis," however, is included a variable remnant of necrosis. Almost invariably, enmeshed in the fibrous connective tissue of the cirrhotic livers, there is still recognizable, in some form, a large or small remnant of the previously necrotic parenchyma of the liver. This varies from definitely recognizable partly or completely necrotic cells to globules of variable size and shape, most of which have a light yellow or greenish-yellow color in paraffin sections stained with hematoxylin and eosin. In frozen sections stained with Sudan IV these globules are pink or red and are also peroxidase positive. The exact nature of these globules has not yet been determined. Treatment of frozen sections for as long as 18 hours with absolute alcohol, methyl alcohol, acetone, ether, petroleum ether, xylene, dioxane, or pyridine does not dissolve out the constituent which takes the stain with the usual Sudan IV (Herxheimer), naphthol Sudan IV (Goldmann), naphthol Sudan black (Lison),[4] and the globules remain in their original form. They are presumably necrotic protoplasmic remnants of the

[3] Not all this number are included in this report.

[4] The Sudan black method of Lison, modified by Miss Ethel Lieb, technician at the Institute of Pathology.

original hepatic cells in which lipoidal material is probably in some form of intimate combination with the protein which interferes with its removal by lipoid solvents The globules, although undoubtedly of cellular origin but not present within cells, give a positive reaction for oxydase (alpha naphthol crystal violet method of Loele) Enmeshed also in the fibrous connective tissue are single cells or small islands of regenerating hepatic cells. These exhibit variation in size of nuclei, some large deeply basophilic nuclei and frequent mitoses. In the fibrous connective tissue of an occasional liver there is some golden yellow pigment, either within the cytoplasm of large mononuclear cells or in the form of granules of amorphous extracellular deposits. Most of this pigment gives the reaction for iron and is probably hemosiderin.

The gross and microscopic pictures of diffuse necrosis of the liver, with or without hemorrhage, have been described in detail and illustrated in a previous paper (1) The changes in these livers are exactly similar and resemble human acute or subacute diffuse necrosis of the liver

Livers with cirrhosis often present in the gross a rough, nodular surface and, in severe cases, a typical "hobnail" appearance (Fig 1), but not infrequently the surface of the liver is relatively smooth and gives no indication of the degree of fibrosis.

Although the necrosis is usually central and midzonal, yet the cirrhosis is mostly periportal However, the alteration of the natural architecture is usually so great that central veins are frequently not easily recognizable The bands of connective tissue are usually very wide and frequently include more than one lobule (Fig 2) The probability is great that the diffuse fibrosis that occurs is a combination of condensation fibrosis as well as a definite increase of periportal connective tissue replacing the degenerated and necrotic parenchyma, but exactly how the fibrosis frequently becomes periportal is not yet quite clear What is clear is that the necrosis evidently precedes the development of the cirrhosis. In this series of animals the multiplication of bile ducts has not been frequent or striking, but in an occasional liver this change has been definite Although the precirrhotic changes are more like those of so called "toxic cirrhosis" or the precirrhotic stage of so called "acute yellow atrophy," yet the final picture is more like that of Laënnec's cirrhosis of both the multilobular and monolobular varieties. There is no exact correlation between the degree of fat infiltration and the degree of cirrhosis in the livers

The kidneys of all rats were not examined, but in those that showed the most severe injury to the liver diffuse necrotizing nephrosis is frequent. The pathological changes in these kidneys differ somewhat from those previously reported (6) in much younger rats fed a choline deficient diet, in which the process was more severe. In these animals the change amounts to diffuse cortical hemorrhagic necrosis, and resembles human bilateral symmetrical cortical necrosis, but in the present series the change is best described as a necrotizing nephrosis.

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[3] Not all this number are included in this report.

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changes, whereas cirrhotic changes, when present, were of a very mild degree and even then were combined with necrosis. Necrosis alone was observed in the livers of 6 of the 17 rats and necrosis with beginning cirrhosis in 2 animals, the incidence, therefore, was still almost 50 per cent as compared with 75 per cent in the control group which received no choline.

In view of the known antagonistic effect of cystine on choline, as well as on casein, in experiments dealing with the lipotropic activity of casein (13), the influence of adding cystine to the diet on the production of hepatic injury was studied in another group of rats (Table II) Daily supplements of 100 mg of *l*-cystine were given to 11 rats In the livers of all these animals signs of more or less severe cirrhosis were seen in the gross specimen and on microscopic examination. Thus it has been proved that cystine is a very potent factor in the accentuation of cirrhosis of the liver in rats.

To another series of 15 rats supplements of 50 mg of *l*-cystine were given daily Pathological changes of the liver, mainly cirrhosis, were manifested by 14 of these rats. Acute diffuse cortical necrosis of the kidneys (6) was found in 4 of these animals.

Cirrhosis of the liver was observed in 3 of 4 rats fed a daily special supplement of 25 mg of *l*-cystine for 150 days. The liver of 1 rat which received only 12 5 mg of *l*-cystine daily for this period showed slight cirrhosis

In conclusion, *l*-cystine appears to exert a definitely injurious effect on the livers of rats maintained on a diet low in casein, high in fat and low in choline. With larger doses of *l*-cystine the hepatic injury seemed to become more accentuated.

The effect of *l*-cystine on the liver was neutralized to a large extent by the daily addition of 10 and 20 mg of choline to diet L II (Table II) Only 4 of the 19 rats in this group exhibited necrotic changes and all 19 remained free from cirrhosis even up to 150 days of the experiment. It is noteworthy that the 4 rats with necrosis of the liver died before the 120th day of the experiment. Cortical necrosis of the kidneys did not occur in this series of animals

A daily special supplement of 0.5 gm. of yeast neutralized the effect of 50 mg of *l*-cystine as well as that of the modified basal diet L II (Table II) The liver of only 1 rat of the 5 rats fed the special supplement exhibited slight cirrhosis during the 150 days of the experiment Excellent growth and completely normal livers were observed in another 5 rats fed daily a combination of 50 mg. of *l*-cystine, 0 5 gm. of yeast, and 10 mg of choline as a special supplement (Table II)

The preventive effect of a supplement of yeast alone on dietary hepatic injury produced by diet L II became apparent even when a low dose of yeast (0 5 gm.) was given three times a week instead of daily (Table II) of a group of 14 rats maintained on the modified basal regime hepatic injury was manifested in only 2 When a daily special supplement of 12.5 mg of *l*-cystine, in addition to 0 5 gm. of yeast three times a week, was fed to 6 rats evidence of cirrhosis was found in the livers of only 2 rats and of necrosis in 1 rat the further addition of 10 mg of choline to the regime of 5 rats prevented the appearance of pathological changes in the liver

A daily special supplement of 20 mg of *dl*-methionine left the incidence of hepatic injury practically unaltered in a group of 6 rats but slightly allayed its severity (Table II) In contrast, special daily supplement of 40 mg of *dl*-methionine had the same prophylactic effect that was produced by cystine plus choline. It is especially interesting that the combined administration of 50 (or 25) mg of *l*-cystine and 20 mg of

EXPERIMENTAL OBSERVATIONS

Diet L II—Twenty rats were put on diet L II in which the casein ratio was reduced from the 18 per cent generally used in investigations on the vitamin B complex (2) to 10 per cent, and 20 per cent of lard was substituted for the usual 8 per cent of

TABLE II

*Results of Feeding 184 Rats Diet L II**

Special supplements						Total No of rats treated	No of rats with no hepatic injury	Hepatic injury present			
l-Cystine daily	Choline daily	*dl* Methionine daily	Liver extract daily	Yeast daily	Yeast 3 times a wk			No of rats	Necrosis	Cirrhosis	Necrosis and cirrhosis
mg	*mg*	*mg*	*gm*	*gm*	*gm*						
—	—	—	—	—	—	20	5	15	2	9	4
—	20	—	—	—	—	17	9	8	6	—	2
—	—	20	—	—	—	6	1	5	1	1	3
—	—	40	—	—	—	8	8	—	—	—	—
—	—	—	0 2	—	—	9	3	6	6	—	—
—	20	—	0 2	—	—	6	2	4	4	—	—
—	—	—	—	—	0 5	14	12	2	2‡	—	—
100	—	—	—	—	—	11	—	11	—	11	—
50	—	—	—	—	—	15	1	14	3	8	3
25	—	—	—	—	—	4	1	3	—	3	—
12 5	—	—	—	—	—	1	—	1	—	1‡	—
50	20	—	—	—	—	8	6	2	2‡	—	—
25	10	—	—	—	—	11	9	2	2	—	—
50	—	20	—	—	—	5	5	—	—	—	—
25	—	20	—	—	—	11	9	2	2‡	—	—
25	10	40	—	—	—	6	6	—	—	—	—
50	—	—	0 2	—	—	11	10	1	—	1‡	—
50	—	—	—	0 5	—	5	4	1	—	1	—
50	10	—	—	0 5	—	5	5	—	—	—	—
12 5	—	—	—	—	0 5	6	4	2	—	1	1
12 5	10	—	—	—	0 5	5	5	—	—	—	—

* All animals except those receiving 0 5 gm of yeast three times weekly were fed diet L II routinely supplemented daily with 20 micrograms of thiamine, 25 micrograms of riboflavin, 20 micrograms of pyridoxine, and 100 micrograms of calcium pantothenate. The rats in the group receiving 0 5 gm of yeast three times weekly were not fed any further vitamin supplements

‡ Only mild changes were observed

butter (Table I) To this modified basal diet the routine daily supplements of B complex factors were added, as described. In the group of 20 rats hepatic injury became an almost regular complication (Table II) Necrosis without cirrhosis was observed in 2 rats, with cirrhosis in 4 rats, and cirrhosis without necrosis in 9 rats that died between the 92nd and the 140th experimental days or were killed on the 150th day

Addition of 20 mg of choline daily reduced the incidence of hepatic injury (Table II) which, in this series of 17 animals, manifested itself almost exclusively by necrotic

changes, whereas cirrhotic changes, when present, were of a very mild degree and even then were combined with necrosis. Necrosis alone was observed in the livers of 6 of the 17 rats and necrosis with beginning cirrhosis in 2 animals, the incidence, therefore, was still almost 50 per cent as compared with 75 per cent in the control group which received no choline.

In view of the known antagonistic effect of cystine on choline, as well as on casein, in experiments dealing with the lipotropic activity of casein (13), the influence of adding cystine to the diet on the production of hepatic injury was studied in another group of rats (Table II) Daily supplements of 100 mg of *l*-cystine were given to 11 rats. In the livers of all these animals signs of more or less severe cirrhosis were seen in the gross specimen and on microscopic examination. Thus it has been proved that cystine is a very potent factor in the accentuation of cirrhosis of the liver in rats.

To another series of 15 rats supplements of 50 mg of *l*-cystine were given daily Pathological changes of the liver mainly cirrhosis, were manifested by 14 of these rats. Acute diffuse cortical necrosis of the kidneys (6) was found in 4 of these animals.

Cirrhosis of the liver was observed in 3 of 4 rats fed a daily special supplement of 25 mg of *l*-cystine for 150 days The liver of 1 rat which received only 12.5 mg of *l*-cystine daily for this period showed slight cirrhosis.

In conclusion, *l*-cystine appears to exert a definitely injurious effect on the livers of rats maintained on a diet low in casein, high in fat and low in choline. With larger doses of *l*-cystine the hepatic injury seemed to become more accentuated.

The effect of *l*-cystine on the liver was neutralized to a large extent by the daily addition of 10 and 20 mg of choline to diet L II (Table II) Only 4 of the 19 rats in this group exhibited necrotic changes and all 19 remained free from cirrhosis even up to 150 days of the experiment. It is noteworthy that the 4 rats with necrosis of the liver died before the 120th day of the experiment Cortical necrosis of the kidneys did not occur in this series of animals.

A daily special supplement of 0.5 gm. of yeast neutralized the effect of 50 mg of *l*-cystine as well as that of the modified basal diet L II (Table II) The liver of only 1 rat of the 5 rats fed the special supplement exhibited slight cirrhosis during the 150 days of the experiment Excellent growth and completely normal livers were observed in another 5 rats fed daily a combination of 50 mg of *l*-cystine, 0.5 gm. of yeast, and 10 mg of choline as a special supplement (Table II)

The preventive effect of a supplement of yeast alone on dietary hepatic injury produced by diet L II became apparent even when a low dose of yeast (0.5 gm.) was given three times a week instead of daily (Table II) of a group of 14 rats maintained on the modified basal regime hepatic injury was manifested in only 2 When a daily special supplement of 12.5 mg of *l*-cystine, in addition to 0.5 gm. of yeast three times a week, was fed to 6 rats, evidence of cirrhosis was found in the livers of only 2 rats and of necrosis in 1 rat the further addition of 10 mg of choline to the regime of 5 rats prevented the appearance of pathological changes in the liver

A daily special supplement of 20 mg. of *dl*-methionine left the incidence of hepatic injury practically unaltered in a group of 6 rats but slightly allayed its severity (Table II) In contrast, special daily supplement of 40 mg of *dl*-methionine had the same prophylactic effect that was produced by cystine plus choline. It is especially interesting that the combined administration of 50 (or 25) mg of *l*-cystine and 20 mg of

dl-methionine daily proved to be beneficial to the liver, although when these supplements were given separately *l*-cystine was injurious and 20 mg of *dl*-methionine was practically without effect The effect of *dl*-methionine given in combination with *l*-cystine is similar to that produced by the simultaneous administration of *l*-cystine and choline

A liver concentrate,[5] which represented the fraction of an aqueous liver extract soluble in 95 per cent alcohol, has been tested as a special supplement to diet L II (Table II) This concentrate was considered by Woolley (14) to be a good source of members of the vitamin B_2 complex other than riboflavin, pyridoxine, and pantothenic acid, as they are needed by the rat Administration of a daily dose of 0 2 gm of this concentrate, alone to 9 rats or together with 20 mg of choline to 6 rats, proved to be completely ineffective in preventing hepatic injury From the additional result that it had a neutralizing effect on *l*-cystine given in doses of 50 mg daily to 11 rats, it could be concluded that in this combination the liver extract acts through its content of choline (or a choline-like substance) Thus these experiments with liver extract furnish new indirect evidence of the importance of the combined administration of cystine plus choline in the dietary management of hepatic injury in rats

Diet S I—If it is assumed that the pathogenesis of dietary necrosis and dietary cirrhosis of the liver is comparable to that of fatty metamorphosis of the liver, as shown by the results of experiments on the lipotropic activity of casein (13), an increase in the content of casein in the experimental diet should have a preventive effect on hepatic injury caused by *l*-cystine similar to that caused by the addition of choline or *dl*-methionine In accordance with this expectation, a special supplement of 50 mg of *l*-cystine daily was given with diet S I, which had a casein content of 18 per cent (Table I), but failed to provoke the slightest pathological change in the livers of 11 rats, even though it was administered for 179 days The diet was routinely supplemented daily with 20 micrograms of thiamine, 25 micrograms of riboflavin, 20 micrograms of pyridoxine, and 100 micrograms of calcium pantothenate

Diet L IV—In order to show that it was not the low content of fat in diet S I which was responsible for the results obtained, a special diet (L IV) was devised with the same relative amount (20 per cent) of fat (lard) as in diet L II but with 18 per cent of casein as in diet S I The routine vitamin B complex supplements were added (Table I) The results obtained with diet L IV were again unequivocal The high intake of casein prevented the appearance of hepatic injury in spite of the simultaneously high intake of lard, regardless of whether no special supplement (10 rats) or a special daily supplement of 50 mg of *l*-cystine (11 rats) was added to the diet.

Diet L III—Decreasing the content of casein in the diet below 10 per cent met with some difficulties Experiments with diet L V, with a casein content of 5 per cent (Table I), showed that it contained apparently too little protein to permit survival of the rats for a period long enough to allow hepatic injury to develop Substitution of corn starch for part of the content of cane sugar and increasing the casein level from 5 to 6 per cent resulted in diet L III (Table I), a slight modification of the ration recommended for special purposes by White and Jackson (15) The experiments performed with diet L III are summarized in Table III The results were essentially

[5] Kindly supplied by Dr David Klein of the Wilson Laboratories, Chicago

the same as those obtained when rats were fed diet L II, with the distinction that the addition of *l*-cystine in a dose as low as 12 5 mg daily had a markedly aggravating effect on the incidence of hepatic injury in 6 of the 7 rats that received this dosage. In all 5 rats that received 25 mg of *l*-cystine daily as a special supplement not only was severe cirrhosis manifested but in 4 of these animals acute necrotizing nephrosis

TABLE III

*Results of Feeding 44 Rats Diet L III**

Special supplement			Total No. of rats treated	No. of rats with no hepatic injury	Hepatic injury present			
l-Cystine daily	Choline daily	Yeast 3 times a wk.			No. of rats	Necrosis	Cirrhosis	Necrosis and cirrhosis
mg	*mg.*	*gm*						
—	—	—	13	3	10	5	4	1
25	—	—	5	—	5	—	5	—
12 5	—	—	7	1	6	—	6	—
25	10	—	6	4	2	2‡	—	—
12 5	10	—	5	4	1	1‡	—	—
—	—	0 5	8	8	—	—	—	—

* For routine daily supplements see note* Table II.

‡ Only mild changes were observed

TABLE IV

*Results of Feeding 62 Rats Diet C I**

Special supplement			Total No. of rats treated	No. of rats with no hepatic injury	Hepatic injury present			
l-Cystine daily	Choline daily	Yeast 3 times a wk.			No. of rats	Necrosis	Cirrhosis	Necrosis and cirrhosis
mg	*mg*	*gm.*						
—	—	—	12	2	10	1	8	1
25	—	—	11	1	10	1	9	—
25	10	—	10	7	3	—	3	—
—	—	0 5	9	7	2	—	2	—
25	—	0 5	10	5	5	—	5	—
25	10	0 5	10	10	—	—	—	—

* For routine daily supplements see note* Table II

was also observed. The beneficial effect of the combined administration of cystine plus choline, as well as of 0.5 gm. of yeast three times a week, could again be clearly demonstrated.

Diet C I —The most severe hepatic changes (necrosis, cirrhosis), often accompanied by ascites and pleural and pericardial effusion, were found in rats fed diet C I (Table I), which had a low content of casein (8 per cent) and high content of fat (crisco, 38 per cent) The increased intake of fat certainly aggravated the manifestation of hepatic injury (Table IV) The effects of the administration of *l*-cystine, on the one hand, and of *l*-cystine plus choline, on the other hand, were fully consistent with the

results characteristic of diets L II and L III Acute necrotizing nephrosis was seen only in the group of rats that received the special supplement of *l*-cystine alone

The results obtained with the use of diet C I, therefore, are a direct confirmation and corroboration of conclusions already firmly established

DISCUSSION

The experiments reported here show conclusively that in the production of dietary hepatic injury (necrosis and cirrhosis, with or without ascites and pericardial and pleural effusion) the determining factors are known to be connected with the lipotropic action of casein (13) In short term experiments identical conditions produce fatty metamorphosis in the liver, whereas experiments of longer duration, up to 150 days, lead to necrosis or cirrhosis of the liver in rats

At the same time, independently of these investigations and the preliminary publication (1) of their results, Webster (16) arrived at similar deductions Results of experiments likewise independently conducted, which were published later by Blumberg and McCollum (17) as well as by Sebrell and his collaborators (18), are in essential agreement with those of the present and preliminary (1) series Fat infiltration of the liver has been considered earlier by Connor (19) a prerequisite of cirrhosis, without correlating this assumption to the phenomenon of the lipotropic activity of casein and to its mechanism.

The lipotropic action of casein is generally (13) believed to be in direct proportion to its content of methionine and to the presence of choline in the diet, but it is reversely influenced by administration of *l*-cystine and supplements of fat, especially cholesterol Identical conditions seem to regulate also the occurrence of cortical hemorrhagic necrosis of the kidneys as it is observed mainly in young rats (6, 20) A thorough review of the experimental data given here reflects mirror-like all the conditional factors in their relation to necrosis and cirrhosis of the liver Thus, the prophylactic effect in rats of a high intake of casein or of adequate supplements of methionine and choline is noted, the latter mainly in combination with cystine Striking is the fact that hepatic injury is caused by supplements of *l*-cystine alone and by a high fat ratio in the diet, which appears to operate here in the same way as in the mechanism of fat infiltration of the liver

One minor discrepancy between the conclusions reached by Blumberg and McCollum (17) and those reported in this and the preliminary paper (1) refers to the rôle of choline in the prevention of hepatic injury According to Blumberg and McCollum addition of choline to the diet (10 mg per gm of diet) resulted in normal livers, whereas in the studies reported here when choline (20 mg per day) was added as a separate supplement to a slightly different form of diet, low in protein and moderately high in fat, hepatic injury, especially in the form of necrosis, still occurred in about 50 per cent of the animals (Table II) It is noteworthy that choline, even when it is given in combination with liver extract (Table II), proved to be ineffective in the prevention of hepatic

injury In contrast, the combined administration of *l*-cystine and choline has been found to be very beneficial in all experiments of the present series. As a matter of fact, this latter result has been confirmed by Blumberg and McCollum (17) as well as by Daft, Sebrell, and Lillie (18)

That choline seems to act through the intermediation of *l-cystine* is also apparent from investigations (21) on hepatic injury in rats following ingestion of dimethylaminoazobenzene (butter yellow) It has been definitely established that choline or *l*-cystine, given separately, exerted no effect on the course of hepatic injury in rats maintained on diet L III when it was supplemented with butter yellow Administration of *l*-cystine *plus* choline, however, afforded definite protection.

The emphasis placed on the combined effect of cystine plus choline is at variance not only with the conclusion of Blumberg and McCollum (17) but also with that of Griffith (20) concerning the rôle of choline in the prevention of cortical necrosis of the kidney Furthermore, the importance of the combined administration of cystine plus choline has hardly been stressed in past work dealing with the phenomenon of the liptropic activity of casein (13) The discrepancy is, of course, not a real one, in view of the fact that in all cases in which a beneficial effect was claimed from choline alone the diet used always contained cystine, even if only in small amounts

The combined effect of *l*-cystine plus choline is reminiscent of a similar phenomenon encountered lately in assays of the growth promoting activity of the essential amino acids which are linked with choline It has been shown by du Vigneaud and his collaborators (22) that homocystine would support growth on a methionine free diet only in the presence of choline or related substances and that methionine, on the other hand, can replace homocystine plus choline According to du Vigneaud and his coworkers, "The explanation presented as the most probable one for this observed relationship of choline to homocystine was that choline had acted as a donor of methyl groups for the synthesis of methionine from homocystine" (23) In these experiments on growth, cystine could not be substituted for homocystine It has recently been demonstrated, however, that methionine may be a precursor of choline in so far as the methyl groups are concerned (22) and that cystine is capable of stimulating growth only when methionine is present in suboptimal amounts (24)

Without being able to give a satisfactory explanation for all these reactions, particularly for the apparent lack of interchangeability of cystine and homocystine, we can say, however, that their relationship to the conditions that determine hepatic injury is evident In this connection it is significant that methionine in adequate doses prevents development of hepatic injury (Table II)[6] just as well as it is prevented by *l*-cystine plus choline, and that suboptimal doses of methionine have the same detoxifying effect on cystine that choline

[6] See also Daft, Sebrell, and Lillie (18)

has, methionine being, probably, a precursor of choline, as stated by du Vigneaud and his collaborators It should be especially pointed out that, whereas with special supplements of liver extract alone (0 2 gm daily) to diet L II the average loss in weight in 150 days in 5 animals was found to be 36 gm and the average gain for 8 rats that received 50 mg of *l*-cystine daily for 150 days was 9 gm , an average gain of 99 gm occurred in a group of 11 rats that received liver extract plus *l*-cystine as special supplements Similar differences were not noted in groups of rats fed special supplements of choline, *l*-cystine and choline plus *l*-cystine, respectively

In conclusion, the impression is gained that the combined administration of choline plus *l*-cystine is necessary for the synthesis of a third substance (methionine?) which in its turn is concerned in the prevention of hepatic injury

It is even more difficult to explain the hepatic injury caused by administration of *l*-cystine alone Griffith (20) attributes it to some unspecific activation of the metabolism This assumption it is not easy to reconcile with the fact that large toxic doses of *l*-cystine may, in a few days or weeks, produce necrosis of the liver (25) and, according to recent investigations, also the specific picture of Laennec's cirrhosis (26) It should be emphasized that in the present experiments and in those of Blumberg and McCollum, as well as of Daft, Sebrell, and Lillie, the amounts of cystine administered as supplements were within the physiological limits of a normal diet. The identical response to toxic doses in short term experiments and to lower doses in investigations of longer duration speaks in favor of a specific effect.

The lipotropic action of casein is generally considered (13) to be completely determined by the interaction of cystine, methionine, and choline Dissenting from this conception, Channon and his collaborators (27) seem to assume the presence of further, until now unidentified, and admittedly less important factors in casein. The experiments here reported have not taken into consideration the amount of cystine and choline administered sufficiently to render any conclusive answer to this problem. It should, however, be mentioned that, at least in the amounts chosen, supplements of *l*-cystine plus choline plus yeast or of *dl*-methionine in large doses were more effective in completely suppressing hepatic injury than was a supplement of *l*-cystine plus choline

Application of the results of these experiments on rats to conditions in man is allowable only within the limitations of a conclusion *per analogiam* Nevertheless, as circumstantial evidence, the unquestionable similarity of the etiological conditions that prevail in the most important form of human cirrhosis, namely, that observed in alcoholics, should be pointed out. Low intake of protein combined with insufficient supply of the vitamin B complex (including choline) is a prominent feature of the daily diet of persons addicted to alcohol and tallies with the leading conditions of dietary cirrhosis in rats Thus, the assumption of a specific injurious effect of alcohol here becomes superfluous

just as it does in pellagra or the beriberi of alcoholics. The recent claim of the beneficial effect of a "highly nutritious diet" supplemented with concentrates of the vitamin B complex (28) on cirrhosis in man is suggestive corroboration of the experiments presented here

It is perhaps also permissible to call attention to the identical nutritional etiology of hemorrhagic cortical necrosis of the kidneys and of necrosis of the liver, and to the occurrence of both pathological lesions in pregnant women, hepatic injury (hemorrhagic necrosis) being often found in eclampsia.

From the point of view of general pathology it is of great interest that identical etiological conditions may lead, in rats, either to necrosis or to cirrhosis of the liver or to both

The experiments reported here have been, thus far, exclusively of a prophylactic nature Their scope needs to be extended to include therapeutic investigations In a few animals in which hepatic injury was ascertained by biopsy, therapeutic administration of yeast or liver powder for a short time (up to 2 months) proved to be insufficient. These experiments are being continued on a larger scale and for prolonged periods.

SUMMARY

Experimental dietary hepatic injury (diffuse or focal necrosis and cirrhosis in rats, with or without ascites and pleural and pericardial effusion) is determined by the dietary factors instrumental also in the production of fat infiltration of the liver and thus opposed to the lipotropic activity of casein. Accordingly, rats maintained on a diet low in casein with a moderately high or high content of fat and without choline regularly exhibited hepatic injury after between 100 and 150 days. Supplements of *l*-cystine had an aggravating effect on the production of cirrhosis of the liver, whereas a supplement of choline alone reduced the severity and the incidence of hepatic injury, although not decisively The combined administration of *l*-cystine plus choline or of *dl*-methionine in adequate doses, however, proved to be highly effective in preventing injury to the liver

These conclusions have been corroborated by the use of different modifications of the basal diet.

Rats with dietary hepatic injury exhibit, in sequence, changes that vary from diffuse necrosis resembling human acute or subacute yellow atrophy, to advanced portal cirrhosis.

Diffuse necrotizing nephrosis was a frequent accompaniment of the hepatic injury Cystine again proved to be a factor which aggravated this condition.

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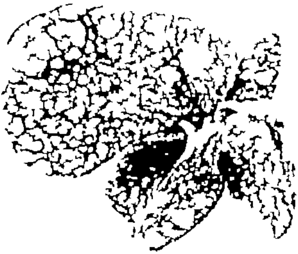
EXPLANATION OF PLATE 12

FIG 1 Cirrhotic liver of rat 6568 (Natural size)

FIG 2 Section of liver of rat 4628, showing moderate degree of cirrhosis and fat infiltration Masson trichrome stain × 71



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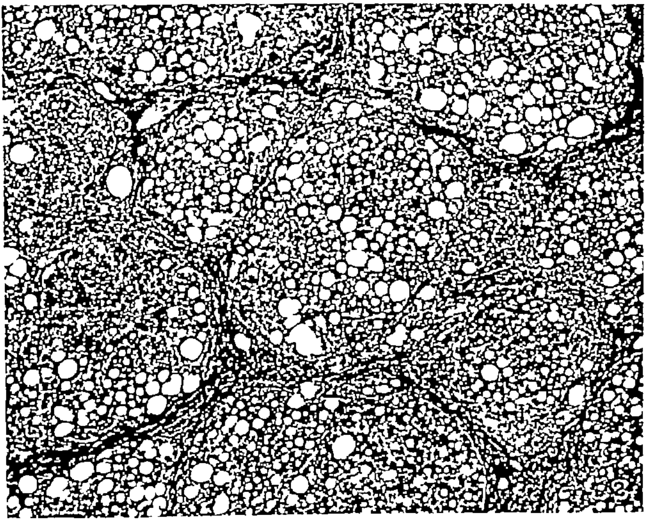

(György and Goldblatt Dietary hepatic injury in rats)

STUDIES ON THE ANTIBACTERIAL ACTION OF THE SULFONAMIDE DRUGS

I. The Relation of *p*-Aminobenzoic Acid to the Mechanism of Bacteriostasis*

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The mechanism whereby sulfanilamide and its derivatives prevent bacterial growth is not known. None of the various theories advanced (1–3) has been conclusively substantiated by direct experimental proof. The most important recent contribution to the problem has been that of Woods (4), who demonstrated that *p*-aminobenzoic acid "blocks" the bacteriostatic effect of sulfanilamide *in vitro*. Following the isolation by Stamp (5) and Green (6) of bacterial extracts which antagonize the action of sulfanilamide, Woods obtained from yeast a potent antisulfanilamide factor which appeared to have chemical properties similar to *p*-aminobenzoic acid but which could not be definitely identified as such. *p*-Aminobenzoic acid itself was found to annul the bacteriostatic effect of sulfanilamide, and Woods predicted that this simple organic acid would eventually be shown to be a metabolite essential for bacterial growth.[1] He also formulated the theory that sulfanilamide causes bacteriostasis by inhibiting specifically the enzymatic reaction involved in the utilization of *p*-aminobenzoic acid and attributed the inhibition of this reaction to the chemical similarity of the drug and the essential metabolite.

Important evidence supporting Woods' hypothesis was published in the same year by Rubbo and Gillespie (8). They reported the chemical isolation from

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[1] To prove a substance essential to bacterial metabolism it is necessary first to demonstrate that it is a growth factor for at least one bacterial species. A growth factor may be defined as an essential metabolite which cannot be synthesized by the bacterial cell and which, therefore, must be added to the culture medium to promote optimal growth. According to views first introduced by Fildes (7), substances known to be bacterial growth factors for one or more species of organism may be considered to be metabolites essential for the growth of many other bacteria and probably of bacteria in general.

yeast of the benzoyl derivative of *p*-aminobenzoic acid and stated in addition that *p*-aminobenzoic acid stimulated the growth of *Clostridium acetobutylicum* in a synthetic medium[2] Their observations led them to conclude that *p*-aminobenzoic acid is a bacterial growth factor and, therefore, an essential metabolite They pointed out, however, that in their experiments with *Cl acetobutylicum* one molecule of *p*-aminobenzoic acid antagonized 23,000 molecules of sulfanilamide This tremendous disproportion between the antagonistic substances was interpreted as strong evidence against Wood's theory that the drug and the essential metabolite compete for the same receptor site on the organism

The data reported in the present paper concern the quantitative aspects of the antagonistic action of *p*-aminobenzoic acid and the more commonly used sulfonamide drugs A detailed study of the quantitative relationship between *p*-aminobenzoic acid and the bacteriostatic effect of sulfanilamide, sulfapyridine, sulfathiazole, sulfadiazine, sulfaguanidine, and diaminodiphenylsulfone has been carried out under rigidly controlled conditions in an attempt to ascertain the manner in which *p*-aminobenzoic acid interacts with these different sulfonamide drugs It was hoped that knowledge of this interaction might lead to a clearer understanding of the mechanism of bacteriostasis and might possibly suggest an explanation for the marked differences in bacteriostatic potency exhibited by the various derivatives of sulfanilamide

Materials and Methods

MacLeod (11) has demonstrated the widespread occurrence of sulfonamide inhibitors in ordinary bacteriological media, and has stressed the importance of using inhibitor-free media in studying the effect of sulfonamide drugs upon bacteria *in vitro* *Bacterium coli* was used in the present studies since it will grow luxuriantly in an inhibitor-free medium containing only asparagine as a source of nitrogen, glucose as a source of carbon, and inorganic salts The addition of *p*-aminobenzoic acid to the medium in concentrations corresponding to those used in the following experiments had no effect upon the growth of the organism as tested by repeated growth curves This fact is of primary importance since the antibacteriostatic effect of compounds which stimulate growth cannot be attributed to a specific action upon the drug, their effect may be due entirely to a non-specific stimulation of the growth of the organism[3]

[2] In attempting to confirm the important findings of Rubbo and Gillespie, both Lampen and Peterson (9) and Park and Wood (10) demonstrated independently that *p*-aminobenzoic acid will function as a growth factor for *Cl acetobutylicum* only in the presence of biotin

[3] The non-specific effect of growth-stimulating substances is well illustrated by experiments reported in the next paper (12)

Culture Medium—The synthetic medium used was prepared in the following manner (13) 5 gm. of NaCl, 2 gm. of K_2HPO_4, 0 5 gm. $MgSO_4$, 2 gm of ammonium citrate, and 3.5 gm of asparagine were added to 1000 cc. of distilled water which had been brought to a boil. After being cooled the mixture was adjusted to a pH of 7 6 with sodium hydroxide (3 to 5 cc. of 2 normal NaOH), and was sterilized in the autoclave. 0 1 cc. of a 10 per cent solution of dextrose was added to the 10 cc. of basal medium in each tube before inoculation. The dextrose solution was sterilized by being heated to boiling for 5 minutes Both the dextrose solution and the basal medium were stored in the ice box.

Strain of B coli—The strain of *B coli communior* employed in the present experiments was obtained through the courtesy of Dr Eleanor A Bliss of the Department of Preventive Medicine of the Johns Hopkins Medical School The organism was originally isolated from the urine of a patient with pyelitis. Daily subcultures were made in synthetic medium and these cultures were used as the source of organism in each experiment The growth obtained in the synthetic medium was remarkably constant.

Inoculum—In all experiments the inoculum employed was 0 1 cc. of a 1 10,000 dilution of a 24 hour culture of *B coli* in synthetic broth. The inoculum, as estimated by plate count, contained approximately 10,000 viable organisms.

Preparation of Stock Solutions of Sulfonamide Drugs and p-Aminobenzoic Acid—In 200 cc. lots of sterile basal medium sulfanilamide and sulfaguanidine were dissolved in concentrations of 0 01 molar, and sulfapyridine sulfathiazole, sulfadiazine, and diaminodiphenylsulfone were prepared in 0 001 molar concentrations. The powdered drug was added in each case to boiling medium and the solution was allowed to cool slowly The maximum concentration of each drug used was limited by its solubility in the basal medium 0 001 molar *p*-aminobenzoic acid in synthetic broth was prepared by the same procedure.

Method of Titration—The antagonistic action of *p*-aminobenzoic acid to each sulfonamide drug was studied quantitatively by titrating the acid against the drug, the appearance of bacterial growth being used as an arbitrary end point. The desired concentrations of the antagonistic substances were attained by adding appropriate amounts of the stock solution of each to plain synthetic broth to make a final volume of 10 cc. 0 1 cc. of 10 per cent glucose was added to each tube just before it was inoculated. The tubes were examined at daily intervals for macroscopic evidence of bacterial growth and the final readings were made at the end of 5 days All cultures were incubated at 37°C.

RESULTS

Data from a typical titration experiment are recorded in Fig 1 It will be noted that over a relatively wide range of sulfapyridine concentrations the minimum amount of *p*-aminobenzoic acid needed to prevent bacteriostasis at the end of 5 days was such that the ratio of *p*-aminobenzoic acid to sulfapyridine was approximately constant [4] As the concentration of drug was decreased,

[4] A constant ratio of *p*-aminobenzoic acid to each sulfonamide drug was noted also at end points determined after 48, 72 and 96 hours as well as at the end of 5 days

however, a point was finally reached where the ratio changed and rapidly approached zero This sudden bend in the titration curve was obviously caused by the drug level's approach to the minimum concentration of sulfapyridine needed to cause bacteriostasis in the basal medium, and it in no way

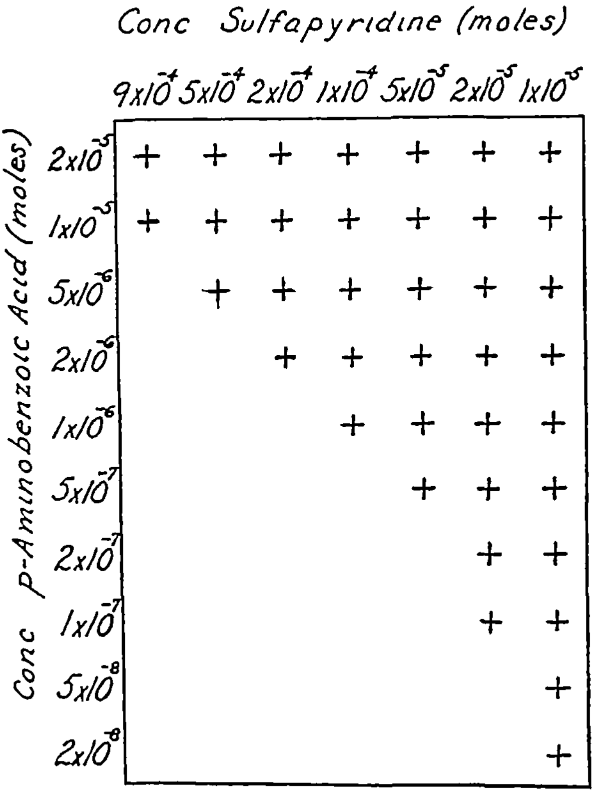

FIG 1 Titration of *p*-aminobenzoic acid against sulfapyridine using bacterial growth as an arbitrary end point. + = visible growth of *B coli* in 10 cc of a synthetic medium within 5 days of inoculation Standard inoculum employed was 10,000 organisms Cultures incubated at 37°C

invalidates the linear relationship exhibited at higher concentrations In the subsequent discussion of titration curves, therefore, only those points will be considered at which the drug level is well above the minimum bacteriostatic concentration

The data summarized in Table I reveal a similar linear relationship between *p*-aminobenzoic acid and each of the sulfonamide compounds studied, the ratio of *p*-aminobenzoic acid to drug remaining approximately constant over

TABLE I

Quantitative Study of the Antagonistic Action of p-Aminobenzoic Acid upon Various Sulfonamide Drugs

Sulfonamide drug	Concentration (molar) Sulf. drug	Concentration (molar) P.a.b.a. needed to prevent bacteriostasis	Ratio: $\frac{\text{Molar conc. P.a.b.a.}}{\text{Molar conc. Sulf.}}$
Sulfanilamide	9×10^{-3}	6×10^{-6}	6.7×10^{-4}
	7×10^{-3}	4×10^{-6}	5.7×10^{-4}
	5×10^{-3}	3×10^{-6}	6.0×10^{-4}
	3×10^{-3}	2×10^{-6}	6.7×10^{-4}
	2×10^{-3}	1×10^{-6}	5.0×10^{-4}
	1×10^{-3}	7×10^{-7}	7.0×10^{-4}
		Average ratio	6.2×10^{-4}
Diaminodiphenyl sulfone	9×10^{-4}	6×10^{-7}	6.7×10^{-4}
	8×10^{-4}	6×10^{-7}	7.5×10^{-4}
	7×10^{-4}	5×10^{-7}	7.1×10^{-4}
	6×10^{-4}	5×10^{-7}	8.3×10^{-4}
	5×10^{-4}	3×10^{-7}	6.0×10^{-4}
	4×10^{-4}	3×10^{-7}	7.5×10^{-4}
		Average ratio	7.2×10^{-4}
Sulfaguanidine	9×10^{-3}	1×10^{-5}	1.1×10^{-3}
	5×10^{-3}	5×10^{-6}	1.0×10^{-3}
	2×10^{-3}	2×10^{-6}	1.0×10^{-3}
	1×10^{-3}	1×10^{-6}	1.0×10^{-3}
	5×10^{-4}	5×10^{-7}	1.0×10^{-3}
		Average ratio	1.0×10^{-3}
Sulfapyridine	9×10^{-4}	1×10^{-5}	1.1×10^{-2}
	5×10^{-4}	5×10^{-6}	1.0×10^{-2}
	2×10^{-4}	2×10^{-6}	1.0×10^{-2}
	1×10^{-4}	1×10^{-6}	1.0×10^{-2}
	5×10^{-5}	5×10^{-7}	1.0×10^{-2}
		Average ratio	1.0×10^{-2}
Sulfadiazine	9×10^{-4}	1×10^{-5}	1.1×10^{-2}
	5×10^{-4}	5×10^{-6}	1.0×10^{-2}
	2×10^{-4}	2×10^{-6}	1.0×10^{-2}
	1×10^{-4}	1×10^{-6}	1.0×10^{-2}
	5×10^{-5}	5×10^{-7}	1.0×10^{-2}
		Average ratio	1.0×10^{-2}
Sulfathiazole	9×10^{-4}	3×10^{-5}	3.0×10^{-2}
	7×10^{-4}	2×10^{-5}	2.9×10^{-2}
	5×10^{-4}	1×10^{-5}	2.0×10^{-2}
	3×10^{-4}	7×10^{-6}	2.3×10^{-2}
	2×10^{-4}	5×10^{-6}	2.5×10^{-2}
	1×10^{-4}	3×10^{-6}	3.3×10^{-2}
	8×10^{-5}	3×10^{-6}	3.7×10^{-2}
		Average ratio	2.8×10^{-2}

Sulf.—sulfonamide.

P.a.b.a.—p-aminobenzoic acid.

the range of each titration experiment This relationship may be expressed mathematically as follows

$$\text{(1)} \qquad \frac{(\text{P a.b a.})}{(\text{Sulf })} = K$$

or

$$\text{(2)} \qquad (\text{P a.b a }) = K\,(\text{Sulf })$$

in which (P.a.b.a.) = molar concentration of *p*-aminobenzoic acid
(Sulf) = molar concentration of sulfonamide drug
K = a constant.

Equation (2) plotted on Cartesian coordinates represents a straight line, with slope K, passing through the origin Since the absolute values for K (as shown in Table I) are extremely small, the titration curves cannot be conveniently plotted arithmetically, their slopes being so small that they practically lie upon the x axis The results of each titration experiment can, however, be graphed on a logarithmic scale as shown in Fig 2 It will be noted that the data thus plotted fall on straight lines parallel to one another and running at an angle of 45° with the axis These lines all approximate very closely the general equation

$$\text{(3)} \qquad \log(\text{P a.b.a.}) = \log(\text{Sulf }) + \log K$$

which will be recognized as the logarithmic variant of equation (2) expressing the same linear relationship between drug and *p*-aminobenzoic acid

Two facts regarding the significance of the constant K deserve special emphasis

(*a*) The constant is equal to the molecular ratio of *p*-aminobenzoic acid and sulfonamide drug at the arbitrary end point chosen for the titration experiments Under standard conditions it defines the amount of *p*-aminobenzoic acid needed to nullify the antibacterial effect of a given quantity of drug

(*b*) The absolute value of K will vary with the type of organism, the size of the inoculum, the conditions under which the organism is grown, and the potency of the bacteriostatic agent employed If all but the last of these variables is kept rigidly constant, as in the present experiments, the magnitude of K becomes an index of the relative bacteriostatic power of the drug tested Under these conditions it is justifiable to refer to K as the "bacteriostatic constant "

As shown in Table II the bacteriostatic constants of the various sulfonamide compounds vary over a relatively wide range of values It will be noted that the more potent the drug the greater is the value of its bacteriostatic constant K, and, likewise, the lower its *p*-aminobenzoic acid titration curve falls on the

logarithmic graph (Fig 2) Thus the bacteriostatic constant of sulfathiazole, the most potent drug studied, is 0 028, whereas that of sulfanilamide, the least active, is only 0 00062

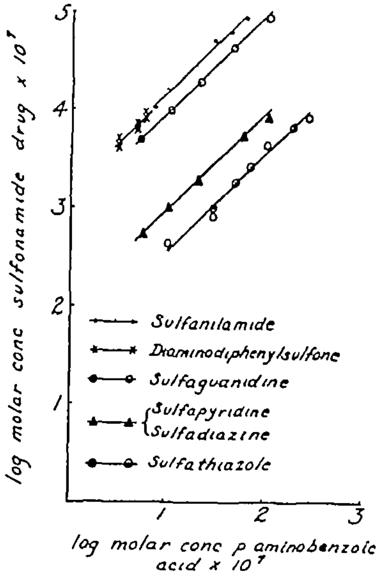

FIG 2 p-Aminobenzoic acid titration curves of sulfonamide drugs. Visible growth of *B coli* (in 10 cc. of a synthetic medium) used as arbitrary end point for each titration. Inoculum standardized at 10 000 organisms and final readings made at the end of 5 days incubation at 37°C Curves plotted for convenience on logarithmic coordinates.

It is of interest to compare the relative values obtained for K with the bacteriostatic potency of the drugs measured by the usual method, *i.e* by determining the minimum concentration needed to cause bacteriostasis in the absence of additional p-aminobenzoic acid. The two sets of values may best be compared by using sulfanilamide as a standard and determining the "sulfanilamide coefficient" as shown in Table II. The data reveal a remarkably close correlation between the sulfanilamide coefficients determined by the two

methods[5] This close correlation is of considerable theoretical significance since it suggests that the bacteriostatic potency of each of the sulfonamide compounds is directly proportional to its ability to neutralize the action of the *p*-aminobenzoic acid added to the culture medium

In summary it may be stated that a quantitative study of the interaction of various sulfonamide drugs with *p*-aminobenzoic acid has revealed the following The antibacterial effect of each sulfonamide compound studied, regardless of chemical structure, was neutralized by the addition of *p*-aminobenzoic acid to the culture medium The amount of *p*-aminobenzoic acid needed to prevent bacteriostasis was directly proportional to the relative bacteriostatic potency of the drug Over a wide range of drug concentrations the minimum amount

TABLE II

Sulfanilamide Coefficients of Various Sulfonamide Drugs Determined Both by Measuring the Minimum Bacteriostatic Concentration and by Titrating with p-Aminobenzoic Acid

Sulfonamide drug	Minimum molar conc of drug causing bacteriostasis	Bacteriostatic constant, K or $\frac{\text{Molar conc. Pa b.a.}}{\text{Molar conc. Sulf}}$ (P.a b.a titration)	Sulfanilamide coefficients	
			$\frac{\text{Min. bact. conc. S}}{\text{Min. bact. conc. X}}$	$\frac{K_x}{K_s}$
Sulfanilamide	0 0004	0 00062	1	1 0
Diaminodiphenylsulfone	0 0002	0 00072	2	1 2
Sulfaguanidine	0 0002	0 001	2	1 6
Sulfapyridine	0 00002	0 01	20	16 1
Sulfadiazine	0 00002	0 01	20	16 1
Sulfathiazole	0 000008	0 028	50	45 2

K, bacteriostatic constant determined by titration with P.a.b.a. (See average ratios, Table 1)

P a b a., *p-aminobenzoic acid.*

Sulf, sulfonamide drug

Min. bact. conc S, minimum molar concentration of sulfanilamide causing bacteriostasis.

Min. bact. conc. X, minimum molar bacteriostatic concentration of sulfonamide drug being compared with sulfanilamide

K_s, bacteriostatic constant of sulfanilamide

K_x, bacteriostatic constant of sulfonamide drug being compared with sulfanilamide.

[5] The *p*-aminobenzoic acid titration method is considered the more accurate of the two since the absolute value for K is obtained by averaging the numerous end points on the titration curve rather than by determining a single end point as in the simple bacteriostasis method. It should be emphasized, however, that neither of these *in vitro* methods can be relied upon at present to estimate the relative therapeutic effectiveness of untried sulfonamide compounds The sulfanilamide coefficients listed above have been measured under highly specialized conditions, namely, in an inhibitor-free medium, it does not necessarily follow that the relative bacteriostatic potency of the drugs will be the same in a more complex culture medium or in the animal body

of *p*-aminobenzoic acid needed to nullify the bacteriostasis was such that the ratio of *p*-aminobenzoic acid to the drug was constant. The *p*-aminobenzoic acid titration curve for each sulfonamide drug thus followed a linear relationship The implications of these facts in relation to the possible mode of action of the sulfonamide drugs are considered significant and will be discussed below

DISCUSSION

The important investigations of Woods on the mechanism of action of sulfanilamide were based on the working hypothesis that "antibacterial substances act by interfering with some substance essential to the bacterial cell" Having demonstrated that *p*-aminobenzoic acid in minute quantities neutralized the bacteriostatic effect of sulfanilamide and sulfapyridine, Woods suggested that the essential substance was *p*-aminobenzoic acid or a closely related compound It is noteworthy, however, that at the time of his publication there was no direct experimental evidence that *p*-aminobenzoic acid was essential to bacterial metabolism, this evidence being supplied later by workers in other laboratories (8–10) Woods suggested further that sulfanilamide and sulfapyridine, because of their close chemical similarity to *p*-aminobenzoic acid, prevent bacterial growth by competing with the latter compound for the bacterial enzyme normally involved in its utilization As evidence for this concept, he pointed out that the slightest deviation from the chemical structure of *p*-aminobenzoic acid greatly reduces the potency of the antisulfonamide effect, just as any change in the *p*-amino structure of sulfanilamide decreases its bacteriostatic properties. In addition he drew attention to other examples in enzyme chemistry of "competitive inhibition" due apparently to similarity of chemical structure. It should be emphasized that Woods' experimental data did not conclusively prove his theory which was presented only as a guide to further investigation.

The results of the studies reported in the present paper add important direct evidence in favor of Woods' theory It was observed that *p*-aminobenzoic acid would neutralize the bacteriostatic properties of all of the six sulfonamide compounds studied, regardless of the differences in their chemical structure, and it was found also that the amount of *p*-aminobenzoic acid needed to prevent bacteriostasis was directly proportional to the bacteriostatic potency of the drug, provided all other variables were held constant. Both of these observations suggest that the bacteriostatic mechanism[4] of the sulfonamide

[4] It is of interest that the sulfonamide portion of the molecule rather than the *p*-amino nucleus appears to be responsible for certain of the toxic reactions caused by these drugs. The $-SO_2NH_2$ radical of sulfanilamide inactivates carbonic anhydrase (14), and this fact has been offered as an explanation for sulfanilamide acidosis (15) Patients becoming sensitive to one sulfonamide drug are often found insensitive to others (16), an observation suggesting that the sensitivity phenomena are caused by some part of the molecule other than the *p*-amino nucleus which is common to all of the compounds

drugs works mainly, if not entirely, through the *p*-amino nucleus which is common to *p*-aminobenzoic acid and to all of the drugs tested The fact that an organism made "drug fast" to sulfathiazole is found to be resistant to the action of other sulfonamide compounds (17) also substantiates this concept In addition, the quantitative study of the interaction of *p*-aminobenzoic acid and the various sulfonamide drugs in bacterial cultures revealed that over a wide range of drug concentrations the minimum amount of *p*-aminobenzoic acid needed to prevent bacteriostasis was such that the ratio of *p*-aminobenzoic acid to drug remained constant Woods previously had observed, in similar experiments, a constant ratio of *p*-aminobenzoic acid to drug when sulfanilamide was added to cultures of *B coli* or *Streptococcus hemolyticus*, and while the present work was in progress Rubbo and Gillespie reported the same observation with sulfanilamide and *Cl acetobutylicum* In none of those experiments, however, were drugs other than sulfanilamide investigated [7]

The linear relationship between drug and *p*-aminobenzoic acid is of considerable theoretical significance since it suggests that bacteriostasis is accomplished through the "competitive inhibition" (19) of an essential enzyme reaction by a substance chemically related to the substrate Assuming the substrate to be *p*-aminobenzoic acid (or a closely related compound) and the inhibitor to be the sulfonamide drug, an equation expressing the same straight-line relationship may be derived on purely theoretical grounds as follows

If *p*-aminobenzoic acid (P) and sulfanilamide, or one of its derivatives (S), compete for the same bacterial enzyme (E), the interaction of P and S with E may be expressed by the equations

$$(1)\quad P + E \rightleftarrows PE \qquad\qquad (2)\quad S + E \rightleftarrows SE$$

Since the concentrations of P and S are infinitely large compared to that of E,[8] it may be assumed that practically all of the available enzyme is bound either by P or S and, therefore, may be considered to be equivalent to $(PE) + (SE)$ The amount of available enzyme is constant at the start of each experiment (constant inoculum of bacteria), so that

$$(3)\qquad (PE) + (SE) = k_1$$

[7] Strauss, Lowell, and Finland (18) studied sulfanilamide, sulfapyridine, and sulfathiazole, and although they concluded that a linear relationship existed between drug and inhibitor, their data do not bear out this conclusion, since the curve obtained for each drug is a straight line only when plotted on semilogarithmic coordinates

[8] The concentration of P and S ranged from 1 to 10^{-5} mg per cc, whereas the concentration of bacteria introduced in the inoculum was 1,000 organisms (or approximately 10 to 12 mg) per cc. Even if it is assumed that the entire bacterial cell functions as active enzyme and that the relatively small *p*-aminobenzoic acid and sulfonamide molecules combine weight for weight with the much larger enzyme molecule, there is still a tremendous discrepancy between the amount of available enzyme and the concentrations of P and S

The ability of the organism to multiply depends upon a certain minimum amount of p-aminobenzoic acid combining with enzyme to form the essential complex PE. At the selected titration end point only the minimum amount of PE needed to initiate growth is present at the start of the experiment, and, therefore, at this point (PE) may be considered a fixed quantity, k_2. It follows that

$$(PE) = k_2 \tag{4}$$

and

$$(SE) = k_1 - k_2 \tag{5}$$

At the titration end point the proportions of enzyme bound by P and S at the start of each experiment will be determined by two factors (*a*) the relative affinities of P and S for the enzyme, E, and (*b*) the relative amounts of P and S in the medium. The affinities of P and S for the enzyme are determined by the chemical properties of the compounds and may be considered constant in any one experiment, although the enzyme affinities of the different sulfonamide drugs probably vary with their chemical structure (12). It follows then that the relative concentrations of PE and SE are directly proportional to the relative concentrations of P and S. That is,

$$\frac{(PE)}{(SE)} = k_3 \frac{(P)}{(S)} \tag{6}$$

and

$$\frac{(P)}{(S)} = \frac{k_2}{k_3(k_1 - k_2)} \tag{7}$$

Since (PE) and (SE) are infinitely small as compared with (P) and (S), the terms (P) and (S) may be considered equivalent to the total amounts of p-aminobenzoic acid (P.a.b.a.) and sulfonamide drug (Sulf) added to the medium.

$$\frac{(\text{P.a.b.a.})}{(\text{Sulf})} = K \tag{8}$$

The last equation given expresses precisely the linear relationship observed experimentally. The close agreement between the theoretical equation and the experimental data adds strong evidence in favor of the assumption that the sulfonamide drugs and p-aminobenzoic acid compete for the same enzyme site on the bacterial cell.[9]

If p-aminobenzoic acid is essential for bacterial growth, and the sulfonamide drugs, through their chemical similarity to this essential metabolite, succeed in blocking the enzyme system normally involved in its utilization, it obviously follows that the bacteria will not grow. The fact that one molecule of p-aminobenzoic acid will antagonize the action of several thousand molecules of drug

[9] Since the completion of this manuscript Wyss (20) has published similar experiments with p-aminobenzoic acid and sulfanilamide and has reached the same conclusion through a different type of mathematical analysis.

does not invalidate this theory as to the mechanism of bacteriostasis, it may only indicate that the essential metabolite has a far greater affinity for the enzyme than has the drug, in which case a great excess of drug will be required to block the essential metabolite from its bacterial enzyme The well known lag in the bacteriostatic action of the drug (21) may be explained by assuming that the supply of *p*-aminobenzoic acid already in the medium (and possibly combined with bacterial enzyme) must become inadequate before the rate of bacterial growth will be noticeably affected

In conclusion it should be emphasized that although the experiments reported in the present paper strongly substantiate Woods' theory as to the mechanism of action of the sulfonamide drugs, the final proof of the theory must await the identification and careful study of the enzyme system (or systems) involved in the utilization of *p*-aminobenzoic acid

SUMMARY

The following observations have been made which substantiate the theory that the sulfonamide drugs used in the treatment of bacterial infections exert their bacteriostatic effect by competing with the essential metabolite, *p*-amino-benzoic acid, for an important enzyme site on the bacterial cell

1 *p*-Aminobenzoic acid was shown to nullify the bacteriostatic effect of all of the six sulfonamide compounds studied even though the drugs exhibited marked differences in chemical structure

2 The bacteriostatic potency of each sulfonamide drug was found to be directly proportional to its ability to counteract the antibacteriostatic action of *p*-aminobenzoic acid

3 In the case of each drug tested over a wide range of concentrations the minimum amount of *p*-aminobenzoic acid needed to prevent bacteriostasis was such that the ratio of *p*-aminobenzoic acid to drug was constant

4 The linear relationship between *p*-aminobenzoic acid and drug was interpreted as indicating the competitive inhibition of an essential enzyme reaction by a substance chemically related to the substrate This interpretation was supported by the fact that the equation derived on purely theoretical grounds relating drug and acid expressed the same linear relationship as that observed experimentally

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STUDIES ON THE ANTIBACTERIAL ACTION OF THE SULFONAMIDE DRUGS

II. The Possible Relation of Drug Activity to Substances Other Than *p*-Aminobenzoic Acid*

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The relation of *p*-aminobenzoic acid to the mode of action of the sulfonamide drugs has been discussed in the preceding paper (1). During the past year the theory has been advanced that sulfapyridine and sulfathiazole prevent bacterial growth by interfering with the functioning of chemically related coenzyme systems. Fildes (2) first called attention to the chemical relation of sulfapyridine to nicotinic acid and of sulfathiazole to thiamin. West and Coburn (3) also noted the similarity of sulfapyridine and nicotinic acid amide and reported *in vitro* experiments with *Staphylococcus aureus* on the basis of which they suggested that sulfapyridine exerts its bacteriostatic effect by interfering with the formation of cozymase from nicotinamide. Dorfman and his associates (4) observed that sulfapyridine inhibited the respiration of "resting" (5) dysentery bacilli and concluded that it acted by disturbing the respiratory function of the chemically related vitamin, nicotinamide. Using pyridine-3-sulfonic acid and its amide, rather than sulfapyridine, McIlwain (6) showed that sulfonic acid derivatives of nicotinic acid, when added to cultures of *Staphylococcus aureus* in a synthetic medium, inhibited the growth promoted by nicotinic acid, nicotinamide, and cozymase.

The attractiveness of the hypothesis that the group attached to the sulfonamide radical interferes with the functioning of certain vitamins and coenzymes is obvious when one considers the structural similarity of the compounds involved. Sulfapyridine, nicotinic acid, nicotinamide, and cozymase each possess a pyridine nucleus. The thiazole ring common to thiamin and cocarboxylase is contained in the sulfathiazole molecule, and sulfadiazine is similarly related to thiamin and cocarboxylase through its pyrimidine radical. Since nicotinic acid, thiamin, and their corresponding coenzymes are known to play important rôles in bacterial metabolism (7), it seems logical to assume that they may be

* This study was supported in part by a grant from The Rockefeller Foundation Fluid Research Fund.

concerned in the bacteriostatic action of the chemically related sulfonamide compounds

To prove the hypothesis, however, it is necessary first to demonstrate that the antisulfonamide effect of the vitamin or its coenzyme is specific in the sense that it antagonizes only the chemically related sulfonamide drug For example nicotinic acid and cozymase should inhibit sulfapyridine but not sulfathiazole, sulfadiazine, or sulfanilamide Thiamin and cocarboxylase, on the other hand, should block the action of sulfathiazole and sulfadiazine but should not influence the action of sulfapyridine or sulfanilamide The experiments reported in the present paper indicate that the antisulfonamide effect of thiamin, nicotinamide, and their respective coenzymes is in no sense specific and is due to stimulation of bacterial growth rather than to a direct antagonistic action upon the sulfonamide drugs These observations are discussed in relation to the mode of action of the complex derivatives of sulfanilamide, and a tentative explanation is offered for the variations in bacteriostatic potency exhibited by the different sulfonamide compounds

Material and Methods

Culture Media—The medium used in all experiments with *Staphylococcus aureus* was one of known chemical composition described by Gladstone (8) The constituents of the medium and the several fractions, which were sterilized separately by autoclaving or filtering, are listed in Table I. All of the labile fractions (part B) were added separately in the order designated. In the experiments with *B coli* the medium used was that described in the preceding paper

Drug, Vitamin, and Coenzyme Solutions—Solutions of the various vitamins and coenzymes[1] used in the bacteriological experiments were prepared by dissolving each substance either in the basal amino acid fraction of the staphylococcal medium or in the synthetic medium for *B coli*, depending upon the organism to be used. The vitamins and coenzymes tested, and the final concentrations in which they were employed, are designated below under each experiment The solutions were sterilized by filtration through Seitz filters Solutions of sulfanilamide, sulfapyridine, sulfathiazole, thionine, and methylene blue were prepared in a similar manner except that instead of being filtered, the basal medium was brought to a boil just before the drug was added and the solution was then allowed to cool slowly without further heating Stock solutions of the sulfonamide compounds and of methylene blue were made up in concentrations of 10^{-2} or 10^{-3} molar, whereas thionine was prepared in a saturated solution The desired concentrations for each experiment were attained by diluting the stock solutions with the appropriate basal medium

Organisms—The strain of *Staphylococcus aureus* used was one isolated from a routine throat culture. The organism was transferred from blood agar to 5 cc. of Gladstone's amino acid medium to which nicotinamide and thiamin had been added in final concentrations of 10^{-5} and 10^{-7} molar respectively Excellent growth was

[1] Cocarboxylase and cozymase were supplied through the courtesy of Merck and Company

obtained in this synthetic medium, and daily subcultures were made by adding 0 1 cc. of a 24 hour culture to 5 cc. of the medium The inoculum used in all experi-

TABLE I

Culture Medium for Staphylococcus aureus

A. Basal amino acid fraction	
KH_2PO_4	4 5 gm.
Water	550 ml.
NaOH 1N	26 ml.
S-Aspartic acid	0 20 gm.
S-Valine	0 15 gm.
S-Leucine	0 15 gm.
S-Alanine	0 10 gm.
S-Glutamic acid	0 10 gm.
S-Iso-Leucine	0 10 gm.
S-Phenylalanine	0 10 gm
S-Lysine hydrochloride	0 10 gm.
S-Glycine	0 05 gm.
L(−) Proline	0 05 gm.
L(−)-Oxyproline	0 05 gm.
L(−)-Tyrosine	0 05 gm.
L(+)-Arginine hydrochloride	0 05 gm.
Amino acids dissolved in the buffer solution made up to 600 ml., adjusted to pH 7 40, tubed in 3 ml. quantities and autoclaved.	
B Labile fractions (added to each tube separately)	
L(−) Cystine, M/200 in N/10 HCl (Seitz filter)	0 10 ml.
NaOH N/5 (autoclave)	0 05 ml.
S-Methionine, M/100 (Seitz filter)	0 10 ml.
L(−) Tryptophane, M/200 (autoclave)	0 05 ml.
Glucose M/2 (Seitz filter)	0 05 ml.
$MgSO_4$ $7H_2O$ M/60 (autoclave)	0 125 ml.
$Fe(NH_4)_2(SO_4)_2$ $6H_2O$ M/500 in N/50 HCl (Seitz filter)	0 125 ml.
Water (autoclave)	1 4 ml.
C. Vitamin fraction	
To make a basal medium for daily subculture and for bacteriostatic experiments, 0.5 cc. of a solution of 10^{-4} M nicotinamide and 10^{-6} M thiamin chloride dissolved in the basal amino acid fraction was added to each tube making final concentrations of 10^{-5} and 10^{-7} respectively	

ments was 0 1 cc. of a 1 10,000 dilution of the daily subculture. Plate counts revealed that the inoculum contained between 10 000 and 20,000 organisms, giving a final bacterial concentration of 2 000 to 4,000 viable organisms per cc. The strain of *B coli* and the manner in which it was cultured in the synthetic medium have already been fully described in the previous paper

EXPERIMENTAL

1 Determination of the Concentrations of Thiamin, Cocarboxylase, Nicotinamide, and Cozymase Optimal for the Growth of Staphylococcus aureus

Knight's experiments (9) to determine the quantities of nicotinamide and of thiamin necessary for the optimal growth of the staphylococcus in Gladstone's synthetic medium were repeated, and it was found that 10^{-6} M nicotinamide (or cozymase) and 10^{-8} M thiamin (or cocarboxylase) would insure heavy growth in 42 hours when growth was estimated by noting the degree of clouding in the culture tubes Growth curves revealed, however, that still higher concentrations of any of these four substances would cause more rapid multiplication of the organism (Figs 1 A, 1 B, and 1 C) Knight's assertion that 10^{-5} M nicotinamide and 10^{-7} M thiamin enabled optimal growth to occur was, therefore, not confirmed in the case of the present strain of staphylococcus That increasing concentrations of nicotinamide, cozymase, thiamin, and cocarboxylase accelerate the growth rate of the staphylococcus is a fact of primary importance in interpreting the antibacteriostatic effect of these compounds to be described below

2 Determination of the Minimum Concentrations of Sulfanilamide, Sulfapyridine, Sulfathiazole, Thiamine, and Methylene Blue That Will Prevent the Growth of Staphylococcus aureus

The minimum bacteriostatic concentrations of the various drugs used were determined by adding increasing concentrations of drug to successive tubes and noting the smallest concentration that would inhibit growth completely for 48 hours.[2] This end point was measured with a reasonable degree of accuracy by allowing the concentrations in consecutive tubes to vary only within relatively narrow limits The concentrations of sulfanilamide in several consecutive tubes were, for example, 6×10^{-4} M, 4×10^{-4} M, 2×10^{-4} M, 1×10^{-4} M, etc. The end points of the sulfonamide drugs were very constant when the same lots of medium and of drug solution were used New medium and new drug solution, when standardized, showed a maximum shift in drug end point of only one tube The dye end points were less constant, showing a tendency to shift one or two tubes in successive experiments To eliminate the significance of any possible variation, the position of the drug or dye end point was checked in all subsequent experiments dealing with bacteriostasis The minimum bacteriostatic concentrations of the various agents tested are listed in Table II

[2] Macroscopically detectable growth

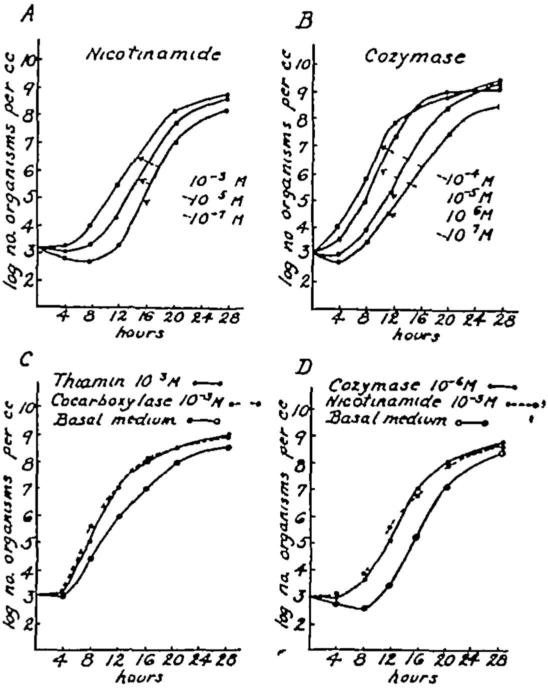


FIG 1

A. Growth curves of *Staphylococcus aureus* in synthetic medium containing increasing concentrations of nicotinamide.

B Growth curves of *Staphylococcus aureus* in synthetic medium containing increasing concentrations of cozymase.

C. Growth curves of *Staphylococcus aureus* in synthetic medium containing high concentrations of thiamin and cocarboxylase.

D Comparison of the growth rates of *Staphylococcus aureus* in 10^{-3} M nicotinamide and in 10^{-6} M cozymase.

3 *The Antisulfonamide Effect of Nicotinamide, Cozymase, Thiamin, and Cocarboxylase*

When *Staphylococcus aureus* was used as the test organism, it was found possible to block[3] the growth-inhibiting effect of sulfanilamide, sulfapyridine, and sulfathiazole with nicotinamide when the latter substance was added to the synthetic medium in a concentration of 2×10^{-3} molar Lower concentrations of nicotinamide blocked more irregularly and a concentration of 10^{-4} molar nicotinamide had no blocking effect whatsoever Cozymase blocked the growth-inhibiting action of these same drugs far more effectively having an antibacteriostatic effect at concentrations between 10^{-6} and 10^{-7} molar (Table III) Thiamin and cocarboxylase, on the other hand, manifested only a barely detectable antidrug effect even at concentrations as high as 10^{-3} molar As suggested by the next experiment, this last result may be explained by the relatively slight degree of growth stimulation brought about by thiamin and cocarboxylase in the synthetic medium as compared to that caused by nicotinamide and cozymase

TABLE II

Minimum Bacteriostatic Concentrations of Sulfonamide Drugs, Methylene Blue, and Thionine As Tested against Staphylococcus aureus in a Synthetic Medium

Sulfanilamide	2×10^{-4} M
Sulfapyridine	8×10^{-5} M
Sulfathiazole	1×10^{-5} M
Methylene blue	1×10^{-6} M (approximate)
Thionine	1 160 dilution of saturated solution (approximate)

TABLE III

Blocking of Bacteriostatic Action of Sulfapyridine and Sulfathiazole by Cozymase

Sulfapyridine			Sulfathiazole		
Drug concentration	Basal medium	Cozymase 10^{-6} M	Drug concentration	Basal medium	Cozymase 10^{-6} M
6×10^{-5} M	+	+	6×10^{-6} M	+	+
8×10^{-5} M	0	+	8×10^{-6} M	0	+
1×10^{-4} M	0	+	1×10^{-5} M	0	+
2×10^{-4} M	0	0	2×10^{-5} M	0	0

+ indicates visible growth of *Staphylococcus aureus* at the end of 48 hours.

In cultures of *B coli* thiamin (10^{-3} M), riboflavin (10^{-5} M), pyridoxine

[3] The term "block" will be used only with reference to the prevention of bacteriostasis.

(10^{-3} M), pantothenic acid (2×10^{-4} M), crystallin biotin (3γ per cc.), crystallin methyl biotin[4] (3γ per cc.), nicotinamide (10^{-8} M) cocarboxylase 10^{-3} M) and cozymase 10^{-4} M) all failed to exert an antisulfonamide effect All of these compounds also failed to increase the antibacteriostatic action of small amounts of *p*-aminobenzoic acid simultaneously added to the medium.

4 Correlation of the Antibacteriostatic Action of Nicotinamide, Cozymase, Thiamin, and Cocarboxylase with Their Ability to Stimulate Bacterial Growth

Instead of influencing only the chemically related drug sulfapyridine, nicotinamide and cozymase were shown to block the bacteriostatic action of all three sulfonamide compounds This observation suggested that the blocking effect might be due to stimulation of growth rather than to a direct antagonistic action upon the drug An attempt was made to correlate the antibacteriostatic effect of nicotinamide and cozymase with their ability to stimulate growth The rate of growth of the staphylococcus in basal medium was compared to that in medium containing from 10^{-8} to 10^{-6} molar nicotinamide or from 10^{-4} to 10^{-7} molar cozymase. It can be seen from the resulting growth curves (Figs. 1 A and 1 B) that the stimulating effect of cozymase is much greater than that of nicotinamide As stated above, the limiting blocking concentration of nicotinamide, *i.e* the lowest concentration that will reverse the inhibition of growth by a sulfonamide drug, is approximately 10^{-3} molar whereas that of cozymase is between 10^{-6} and 10^{-7} molar If the growth rates of the staphylococcus in 10^{-3} molar nicotinamide and in 10^{-6} molar cozymase are plotted on the same graph (Fig 1 D), it can be seen that they are approximately the same, indicating a close correlation between the growth promoting properties and the drug blocking effect of the compounds.

Similar experiments were also carried out with *B coli* grown in the synthetic medium described in the preceding paper Neither nicotinamide nor cozymase is a growth factor for *B coli* as each is for *Staphylococcus aureus*, and repeated growth curves showed that cozymase did not stimulate the growth of *B coli* in the synthetic medium. Failing to stimulate growth, cozymase, even in concentrations of 10^{-4} molar, failed also to block the action of any of the sulfonamide drugs. It appears, therefore, that the antibacteriostatic action of nicotinamide and cozymase is dependent upon acceleration of bacterial growth rather than upon a direct antidrug effect.

5 Lack of Specificity in the Antibacteriostatic Effect of Cozymase

Quantitative experiments were carried out to determine whether or not the blocking effect of cozymase was greater against sulfapyridine to which it is

[4] Obtained through the courtesy of Professor V du Vigneaud, Cornell University Medical School.

chemically related than against sulfanilamide or sulfathiazole to which it is unrelated The degree of blocking by cozymase of each of the compounds was found to be approximately the same (Table III)

To determine whether or not the antibacteriostatic action of cozymase bore any specific relation to the sulfonamide group of drugs, cozymase was titrated against thionine and methylene blue Both of these compounds are dyes which probably achieve their bacteriostatic effect by altering the oxidation-reduction potential of the culture medium (10), a mechanism apparently quite different from that by which the sulfonamide drugs act (1) Cozymase blocked the growth-inhibitory effect of thionine regularly Less satisfactory results were obtained with methylene blue because of the instability of the bacteriostatic end point of this dye, but the general result was the same as with thionine These experiments are compatible with the view that the drug-blocking effect of cozymase is unrelated to the chemical structure of the drug involved and merely results from the stimulation of bacterial growth

6 The Failure of Sulfanilamide, Sulfapyridine, and Sulfathiazole to Affect the in Vitro Action of Cocarboxylase As a Coenzyme

Cocarboxylase is known to function as a coenzyme in the decarboxylation of pyruvate by the enzyme carboxylase contained in yeast The activity of cocarboxylase may be conveniently estimated by measuring manometrically in the Warburg apparatus the rate of evolution of CO_2 from a carboxylase-cocarboxylase-pyruvate mixture The method employed in the present studies was that of Lohmann and Schuster (11), the reaction being carried out at a pH of 6 6[5] The possible inhibitory action of sulfanilamide, sulfapyridine, and sulfathiazole upon the functioning of cocarboxylase was tested by carrying out the reaction in the presence of a final concentration of 10 mg per cent of drug Although sulfathiazole, which is chemically related to cocarboxylase, was added in concentrations 50 to 200 times greater than that of the coenzyme, it failed to influence the reaction Sulfanilamide and sulfapyridine likewise were without effect. In preliminary unpublished experiments with sulfapyridine and the cozymase-apozymase system, Walti also failed to detect any inhibitory effect of the drug upon the action of the coenzyme (12)

DISCUSSION

Direct evidence has been presented in the preceding paper supporting the theory that the sulfonamide drugs prevent bacterial growth by interfering

[5] The dried yeast preparation used as a source of carboxylase and the sodium pyruvate were supplied through the courtesy of Dr Otto Bessy of the Department of Biological Chemistry, Harvard Medical School. The authors are grateful to Dr C L Gemmel of the Department of Physiology, Johns Hopkins Medical School, for the Warburg apparatus used in these studies

with the metabolic function of *p*-aminobenzoic acid According to this theory there are at least two possible explanations for the fact that some of the more common derivatives of sulfanilamide are considerably more potent bacteriostatic agents than the parent drug First, the greater bacteriostatic powers of the substituted sulfonamide compounds, such as sulfapyridine, sulfathiazole, and sulfadiazine, may be explained by assuming that the radical attached to the sulfonamide group interferes with the metabolism of a second substance essential to the bacterial cell, just as the *p*-amino nucleus of these compounds apparently disturbs the function of *p*-aminobenzoic acid Such a dual effect on the part of the more complex derivatives of sulfanilamide might well account for their increased bacteriostatic potency Second, the greater antibacterial powers of the substituted sulfonamide compounds may be explained by assuming that the chemical group attached to the sulfonamide radical enables the compounds to interfere with the metabolism of *p*-aminobenzoic acid more effectively than does the simpler sulfanilamide molecule. According to this second hypothesis the only metabolic function of the bacterial cell interfered with by the sulfonamide compounds is that concerned with the utilization of *p*-aminobenzoic acid, the degree to which this function is disturbed determining the relative bacteriostatic potency of the drug

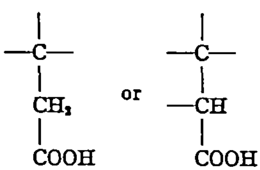
The first of these hypotheses has received support in the publications of Dorfman and his coworkers and of West and Coburn, who have advanced the view that sulfapyridine specifically alters the metabolism of the pyridine-containing coenzyme, cozymase On purely theoretical grounds the same reasoning might be applied to sulfathiazole and sulfadiazine since both of these compounds are structurally similar to thiamin and its coenzyme, cocarboxylase The results of the experiments reported in the present paper however, fail to confirm the view that the chemical group linked to the sulfonamide radical plays a specific rôle in interfering with either the formation or the function of the analogous coenzyme. It has been shown that the antisulfonamide effect of cozymase, emphasized by West and Coburn, is in no sense specific but is due to its action as a growth stimulant rather than to a directly antagonistic action against the drug[4] Also the *in vitro* activity of the coenzyme, cocarboxylase, was found to be unaffected by the chemically related sulfonamide compound, sulfathiazole, even when the latter was present in concentrations 200 times greater than the coenzyme. Both of these observations cast grave doubt upon the attractive theory that the bacteriostatic effect of sulfapyridine, sulfathiazole, and sulfadiazine is related to their structural

[4] It should be pointed out that *p*-aminobenzoic acid blocks efficiently the sulfonamide drugs in a medium in which it fails to stimulate growth (1) The dissociation of growth stimulation and antidrug effect would appear to be fundamental in determining whether a given chemical compound is specifically related to the mechanism of bacteriostasis

similarity to nicotinamide, thiamin, and the respective coenzymes The results likewise fail to substantiate the view that the greater antibacterial power of the more complex sulfonamide drugs is due to a dual effect upon the metabolism of the bacterial cell as compared to the single effect exerted by the simpler sulfanilamide molecule

The second hypothesis advanced to explain the differences in bacteriostatic potency exhibited by the various common sulfonamide drugs is, on the other hand, entirely compatible with the observations reported in the previous paper *p*-Aminobenzoic acid was shown to block the bacteriostatic effect of all of the sulfonamide compounds studied, regardless of their chemical structure, and the bacteriostatic potency of each drug was found to be directly proportional to its ability to nullify the blocking effect of *p*-aminobenzoic acid Both of these observations suggest that all of the sulfonamide drugs studied cause bacteriostasis by interfering with a single metabolic function of the bacterial cell, namely that concerned with *p*-aminobenzoic acid

Evidence has been presented also that the drugs exert their bacteriostatic effect by competing with *p*-aminobenzoic acid for the enzyme system normally involved in its utilization If the mechanism of bacteriostasis concerns only the competitive inhibition of this particular enzyme system, it must be assumed that the relative bacteriostatic power of a given sulfonamide drug depends upon its relative ability to disturb the function of this essential system Such an assumption is not without foundation for analogous variations in competitive inhibition by related chemical compounds are common in enzyme chemistry For example, Quastel and Wooldridge (13) have shown that various organic acids having in common the structure



inhibit the important respiratory enzyme, succinic dehydrogenase The authors have attributed the inhibition to the structural similarity of these compounds and the specific substrate, succinic acid, and have postulated that the common chemical configuration causes the inhibitors to become adsorbed on to that part of the enzyme surface which normally adsorbs and activates the substrate Quastel and Wooldridge have demonstrated in addition that all of the related acids do not cause the same degree of inhibition, the inhibitory effect being quantitatively different in the case of each compound, presumably because of a specific degree of affinity for the enzyme Thus the present hypothesis advanced to explain the mode of action of the sulfonamide drugs may be considered exactly analogous to the competitive inhibition of succinic dehydrogenase, for the experimental evidence strongly suggests that

sulfanilamide and its derivatives, due to their chemical similarity to *p*-amino-benzoic acid, competitively inhibit the enzyme involved in its utilization and that different sulfonamide drugs inhibit this essential enzyme reaction in different degrees.

To prove conclusively this "unitarian theory" as to the mechanism of sulfonamide bacteriostasis it will be necessary first to identify the enzyme system that utilizes *p*-aminobenzoic acid and secondly to demonstrate that the relative bacteriostatic power of a given sulfonamide drug is directly proportional to its ability to inhibit this particular enzyme system An attempt is now being made to identify the enzyme (or enzymes) involved in this apparently vulnerable cycle of bacterial metabolism

SUMMARY

1 In cultures of *Staphylococus aureus* in a synthetic medium nicotinamide and cozymase were shown to block the bacteriostatic action of chemically unrelated sulfonamide drugs as well as the chemically related compound sulfa pyridine. The antibacterial properties of organic dyes totally unrelated to the sulfonamide compounds (methylene blue and thionine) were also nullified by the addition of cozymase to the culture medium.

2 The antagonistic action of the pyridine-containing coenzyme, cozymase, was found, by quantitative study, to be no greater against sulfapyridine than against other structurally dissimilar sulfonamide compounds.

3 The antidrug effects of nicotinamide and cozymase in staphylococcus cultures were observed to be directly proportional to their ability to stimulate the growth of the organism in the synthetic medium. When tested in cultures of *B coli* in which they failed to accelerate bacterial growth, these same substances failed to influence the bacteriostatic action of the sulfonamide drugs.

4 The *in vitro* action of the coenzyme, cocarboxylase, as measured in the Warburg respirometer, was shown to be unaffected by the chemically related drug, sulfathiazole, even when the latter was present in great excess.

The above observations fail to support the theory that sulfapyridine, sulfa thiazole, and sulfadiazine prevent bacterial growth by interfering with the functioning of the chemically related coenzymes, cozymase, and cocarboxylase The mode of action of sulfanilamide and its more common derivatives is discussed in the light of these observations, and a tentative theory is offered to explain the differences in bacteriostatic potency exhibited by the various sulfonamide compounds.

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STUDIES ON THE PURIFICATION OF POLIOMYELITIS VIRUS

I. YIELDS AND ACTIVITY OF PREPARATIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION*

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The application of high speed differential centrifugation to the purification of poliomyelitis virus has been a logical step since Stanley and Wyckoff (1) showed that relatively small and unstable plant viruses could be isolated as homogeneous materials by this method The sedimentation of poliomyelitis virus in an ultracentrifuge was first demonstrated by Schultz and Raffel (2), and more recently Clark, Rasmussen, and White (3) obtained sediments which showed an irregular but in some instances high virus activity While it has been shown that high speed centrifugation resulted in a sedimentation of the virus, evidence has not been provided that other macromolecular materials associated with normal tissue were not also sedimented along with the virus under these conditions. In view of the work of several investigators (4–6) on the concentration of macromolecular materials from various normal animal tissues, such constituents might very well be present together with virus in the high molecular weight fractions obtained from poliomyelitis-infected tissues.

This paper presents the results of experiments on the application of differential centrifugation to the extracts of glycerolated or frozen, infected medullae-cords from *rhesus* monkeys and to similar extracts of glycerolated or frozen, normal medullae-cords. The results show that when extracts of infected as well as normal cords, that have been stored in glycerol, are subjected to high speed centrifugation, appreciable yields of high molecular weight substances are obtained. However, when normal tissue that had been frozen and stored for appreciable lengths of time was used, no significant amount of high molecular weight, nitrogen-containing compounds was recovered. Frozen infected tissue, on the contrary, yielded small but significant amounts of high molecular weight material possessing both a high and uniform virus activity

EXPERIMENTAL

Purification of the virus by differential centrifugation requires (1) the preparation of large quantities of infectious extract free from tissue fragments

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

and lipoidal material, (2) the separation of the virus from soluble constituents of low molecular weight, and (3) the separation of the virus from high molecular weight substances which are thrown down with the virus in the ultracentrifuge The first step was accomplished by suspending the infected cords in buffer by means of a Waring blendor, by centrifuging the tissue suspension at 3800 R P M in a large angle centrifuge, by extracting the supernatant liquid with ether, and then by filtering the aqueous extracts through celite The virus was sedimented, and aggregated colloidal material was removed by centrifugation in an air-driven centrifuge of the improved Pickels' design[1] (7) The virus used was the M V strain mixed with a strain provided by Dr Charles Armstrong in 1936 and carried in monkeys since then by Dr E W Schultz The details of the procedure which was applied both to infected and normal tissues are as follows

The spinal cord and medulla from each animal was removed aseptically and either dropped into a 1-1 glycerol-0 1 M phosphate buffer mixture at pH 7 and stored in a refrigerator or placed in a large test tube (25 × 200 mm.) and stored in a freezing chamber at −10°C About 100 gm. of the pooled glycerolated tissue, drained free of the glycerol-phosphate solution, or the same amount of the frozen tissue, and 200 ml. of buffer cooled to 0°C were mixed in the blendor for 3 minutes During this time, the temperature of the mixture increased to about 15°C, and it was necessary to cool the container in ice before mixing was continued In this way the suspension was mixed for a total of about 15 minutes It was then transferred to 100 ml. centrifuge bottles and centrifuged at 3800 R.P M. in an angle centrifuge in a room maintained at 2°C until a relatively clear supernatant fluid was obtained. The time necessary varied with the solvent used. About 1½ hours were necesssary when Ringer's solution was used and about 3 to 4 hours when water or 0 05 M phosphate or 0 01 M borate buffer was used. The supernatant fluid was pipetted off, and the residue again transferred to the blendor and extracted as before with an additional 100 ml. of ice-cold solvent for about 3 minutes This suspension was centrifuged in the angle centrifuge, and the supernatant fluid removed as before. In this way a third, fourth, and in many cases a fifth extract was prepared.

The extracts, which at this stage were milky in appearance, were combined and extracted in a separatory funnel with one-half a volume of ether An emulsion was obtained which was broken in the angle centrifuge into an ether layer, a middle layer of semisolid gelatinous material, and a clear aqueous bottom portion The aqueous layer was removed, and the semisolid material re-extracted once with buffer The amount of this semisolid material varied greatly with the buffer used in preparing the original extract. It was decreased to a thin skin-like layer in each centrifuge tube when Ringer's or physiological salt solution was used but was as thick as a centimeter when the tissue was extracted with water or dilute buffer The extracts were now almost water clear with the exception of a few particles of solid which were removed by filtration through a thin layer of a mixture of approximately equal

[1] The authors are indebted to Dr E G Pickels for providing photographic copies of scale drawings of the driving mechanism.

parts of Hyflo and Standard supercel. The filtrate from either infected or normal cords was a clear liquid usually slightly pink in color due to the presence of small amounts of hemoglobin. That from poliomyelitic tissues was infectious in about 100 per cent of the monkeys injected intracerebrally with 1 ml. of a 1 to 1000 dilution based on the weight of cords extracted.

A volume of the clear filtrate equal to 112 ml., in eight stainless steel tubes was centrifuged at 600 R.P.S. for 1½ hours in a dowmetal head similar in design to that described by Wyckoff and Lagsdin (8) The head was cooled to 0°C at the beginning of a run and warmed up to about 10°C after 1½ hours. The average centrifugal force was about 79 000 × gravity After a centrifugation run the supernatant liquid was carefully pipetted off and replaced with an equal amount of the clear extract, which was in turn centrifuged at 600 R.P.S. In this way the high molecular weight

TABLE I

Examples of Analyses of Supernatant Liquids after Successive Sedimentations of High Molecular Weight Constituents at 600 R.P.S.

Experiment No.	Amount and type of starting material	Total nitrogen in					
		Original extract	Supernatant liquids from successive centrifugations				
			First	Second	Third	Fourth	Fifth
		mg	mg	mg	mg	mg	mg
6	100 gm. glycerolated virus cords		269	0 63	0 11		
19	88 gm. frozen normal cords	245	229	3 4	0 80	0 30	0 26
20	85 gm. frozen virus cords	272	268	1 74	0 36	0 14	0 035
22	44 gm. frozen virus cords	187	180	1 67	0 47	0 19	0 14
23	103 gm. frozen virus cords	205	196	1 23	0 16	0 031	0 011

With the exception of Experiment 22, the pooled tissues were obtained from *rhesus* monkeys. In Experiment 22 the tissues were obtained from *cynomolgus* monkeys. The solvents used were as follows in Experiment 6, 0 05 M phosphate at pH 7.5, in Experiments 19 and 22, 0 01 M borate at pH 7.8, and in Experiments 20 and 23 Ringer's solution. Nitrogen determined by method of Levy and Palmer (9)

materials in the extract from 100 gm of tissue and in some cases from 200 gm. was concentrated into eight tubes. The sediments were combined and resuspended in about 28 ml. of solvent. Aggregated colloidal particles were removed from this solution in the air-driven centrifuge by running it at a speed of 500 R.P.S. for a few minutes. The supernatant fluid and the wash liquid from the sediment obtained after a similar run up and down were then subjected to a second sedimentation run at 600 R.P.S. for 1½ hours The supernatant liquid was pipetted off the sediment resuspended in buffer and aggregated material again removed as before.

Analyses of the supernatant liquids from each successive sedimentation showed that the amount of unsedimentable nitrogen decreased and was insignificant after the fourth sedimentation. Most preparations were, therefore, subjected to four centrifugal cycles of purification before they were considered free from low molecular weight constituents. Examples of the amounts of nitrogen present in successive supernatant liquids from extracts of infected and normal tissues when phosphate, borate, or Ringer's solution was used are shown in Table I

The sediment obtained after four sedimentations was dissolved in a small volume of solvent, the solution was centrifuged at slow speed to remove any insoluble residue, and an aliquot was analysed for total nitrogen by the method of Levy and Palmer (9) Aseptic technique was used throughout the purification procedures and the temperature was kept as near 0°C as possible. The time required for the preparation of the extracts and for the purification of the high molecular weight constituent from 100 gm. of tissue averaged about 10 days

Recovery of Virus Activity after Ultracentrifugation

To determine the efficiency of the above described method in sedimenting the virus and to obtain a rough measure of the loss of virus in the discarded supernatant liquids, a sample of infectious filtrate was subjected to three centrifugal cycles of purification. This involved sedimentation, removal of the supernatant liquid and re-solution of the sedimented material in fresh solvent The activity of the sediment was compared with that of a similar quantity of filtrate, which had been treated in the same way with the exception that the sediment was redissolved each time in the original supernatant liquid The results found in three experiments are shown in Table II

Activity Tests—In all cases in which a preparation was tested for virus activity, 1 ml. of the dilution or concentration tested was injected intracerebrally into *rhesus* monkeys under ether anesthesia The dilutions were prepared immediately before inoculation in the same buffer used for the extraction of the infected tissues After injection the animals were observed daily for tremors and the onset of a flaccid paralysis Those which developed paralysis were sacrificed within a day or two after the paralysis had become extensive In a few cases in which animals died during the incubation period without showing paralysis, histological examinations were made of the medulla and cord. If typical lesions were found, the test was considered positive Animals which failed to develop poliomyelitis within 3 weeks after injection were counted negative, and were used again in later experiments These monkeys almost without exception finally came down with poliomyelitis

It can be seen that the amount of virus activity present in the sediment obtained after one or three centrifugal cycles of purification was approximately the same as that of the original extract or of the control in which the sediment was redissolved each time in the original supernatant liquid The amount of virus aggregated because of packing in the centrifuge or lost in the discarded supernatant liquids was not sufficiently great, therefore, to be detected by these infectivity measurements Several experiments on the activity of the supernatant liquids obtained after a run at 600 R P S gave results which led to the same conclusion The supernatant liquid was infectious in only a small percentage of the animals injected with doses of 1 ml Before ultracentrifugation, 1 ml of the clarified extracts was usually infectious at a dilution of about 1 120

Yields of High Molecular Weight Materials from Glycerolated and Frozen Infected and Normal Tissues

The yields of sedimentable nitrogen obtained from poliomyelitis-infected and normal cords when subjected to the above described procedures are

TABLE II

Recovery of Poliomyelitis Virus Activity after Centrifugation at 600 R.P.S for 1½ Hours

	Dilutions (based on weight of tissue extracted)			
	1 500	1 1000	1 2000	1-4000
1 Original extract.				
Experiment 1	++*	++	+−	+−
2. Original extract centrifuged and sediment resuspended in supernatant liquid				
Experiment 1	++	+−	−−	−−
2	++	+−	++	+−
3 Original extract centrifuged, supernatant liquid discarded, and sediment resuspended in volume of buffer equal to that of supernatant liquid.				
Experiment 1	++	+−	++	−−
2		+−	+−	−−
4. Once-sedimented virus treated two additional times as in 2				
Experiment 1	++	+−	++	++
2	++	+−	−−	+−
" 3	++	+−	++	
5 Once-sedimented virus treated two additional times as in 3				
Experiment 1	++	+−	++	+−
" 2	++	+−	+−	+−
3	++	++	++	

In Experiments 1 and 2 Waring blendor extracts before ether extraction were used. In Experiment 3 the extract was further clarified by ether extraction, etc. In Experiments 1 2, and 3 the buffer consisted of 0.01 molar borate at pH 7.8 distilled water and Ringer's solution without glucose respectively

* Each + sign indicates that the monkey developed poliomyelitis. Each − sign indicates that the monkey failed to develop poliomyelitis within 3 weeks, but later either proved susceptible or died from other causes.

shown in Table III. Data are included for tissues that had either been stored in glycerol at 5°C or kept in the frozen state at −10°C To provide a measure of the length of time the tissues were stored in each experiment, the average age of the pool is given. This was obtained by dividing the total length of time all the cords in a pool were stored by the number present. The number of

cords, in most experiments, and the dates they were removed and the extracts prepared are as follows

Experiment 7—Aug 15, 1938, to Nov 18, 1939 Extracted Apr 22, 1940, Experiment 11 May 15 to June 2 Extracted June 19, 1940, Experiment 16 Aug 9

TABLE III

Yields of High Molecular Weight Constituents from Infected and Normal Tissues

Type of tissue	Method of storage	Experiment No *	Average length of time tissue was stored	Time required for purification	Amount of tissue ground	No of sedimentations	Yield (nitrogen)	Yield nitrogen per 100 gm. tissue
			days	*days*	*gm*		*mg*	*mg*
Infected	In 50 per cent glycerol-phosphate at 5°C	7	386‡	30	107	4	0 97	0 91
		11	26	20	195	3	0 95	0 49
		16	42	8	92	3	2 1	2 3
		17	140	77	85	4	0 57	0 67
		27	283	11	209	3	0 98	0 47
Infected	In stoppered tubes without solvent at −10°C	20	81	6	85	5	0 15	0 18
		21	145	8	70	3	<0 01	<0 01
		22	7	8	44	5	0 51	1 16
		23	85	9	103	5	0 078	0 076
		24	105	10	170	3	0 12	0 071
		26	30	12	186	3	0 19	0 10
		28	9	8	170	3	1 0	0 59
		29	13	17	73	4	0 16	0 22
		30	19	8	93	3	0 39	0 42
Normal	In 50 per cent glycerol phosphate at 5°C	31	54	13	57	3	0 22	0 39
Normal	In stoppered tubes without solvent at −10°C	19	4	10	70	5	2 74	3 91
		25	54	10	111	3	0 04	0 036
		32	34	4	112	3	0 18	0 16

* Experiments 19, 20, 22, and 23 are the same experiments referred to in Table I The solvents used in the other experiments were as follows 7, 11, and 16, 0 05 M phosphate at pH 7.5, 17, 0 01 M sodium borate at pH 7 8, 21, Ringer's solution, in all other experiments Ringer's solution without glucose

‡ The cords in this pool were collected in connection with earlier studies carried out by Professor E W Schultz

to Oct. 14, Extracted Oct 23, 1940, Experiment 17 June 1 to July 8 Extracted Nov 9, 1940, Experiment 19 One each on Feb 7 and 9, three on Mar 19, and five on Mar 20 Extracted Mar 21, 1941, Experiment 20 One each on Dec. 4, 5, 13, 27, 28, and 30, 1940, and Mar 7, 1941, and two each on Jan 6 and Mar 6 Extracted Apr 4, 1941, Experiment 21 One each on Nov 17, 28, and 29, and three each on Nov 16 and 20, 1940 Extracted Apr 15, 1941, Experiment 22 Three on Apr

21 and five each on Apr 20 and 22 Extracted Apr 28, 1941, Experiment 23 Five on Nov 13, 1940, one each on Mar 8 9, 11, and Apr 17, two on Apr 19, and four on Apr 20 Extracted May 14, 1941 Experiment 24 Nine on Nov 14 and three on Nov 15, 1940, one each on May 8, 10, 12, 16, and 18, three on May 14, two each on May 15 and 17 Extracted May 29, 1941, Experiment 25 One each on Mar 25, Apr 16, 22, and June 10, two on May 24 and four on July 15 Extracted July 21, 1941, Experiment 26 May 21 to July 22 Extracted July 25, 1941, Experiment 27 One each on Oct. 15 16 24, 25, 26, Nov 8 9, and 11, two each on Oct. 21 and Nov 4, and four on Nov 12 1940 Extracted Aug 11, 1941, Experiment 28 One each on July 23 Aug 6, 22 23 and 26, two each on Aug. 24 and 25 three on Aug 8 and five each on Aug 14 and 16 Extracted Aug 27, 1941 Experiment 29 Five each on Aug 26 and 27 Extracted Sept. 9, 1941, Experiment 30 One each on Aug 26 and Sept 19, three on Sept. 20, and six on Sept. 22. Extracted Oct. 8, 1941, Experiment 31 One each on June 24, July 18 19, and Oct. 8, two on Oct. 7 Extracted Oct. 21, 1941, Experiment 32 One each on Aug 14 and Nov 4, two each on Aug 4 Oct. 9 and 28, three on Oct. 10, and four on Oct. 30 Extracted Nov 11, 1941

Appreciable amounts of high molecular weight materials were obtained from both glycerolated normal and infected tissues regardless of the length of time the tissues were stored. The results with frozen tissues, in general, show a striking decrease in the amounts of high molecular weight substances present. It is evident, however, that the yield varies, depending on the period of storage In Experiments 22 and 28, in which the cords had been stored for only a short time relatively large quantities of high molecular weight materials were obtained. In contrast to these, in Experiments 21, 23, and 24, in which the tissues had been kept for from 2 to 5 months on the average, only small yields of sedimentable nitrogen were isolated. As shown in Experiments 19, 25, and 32, similar results were obtained with normal tissue stored at $-10°C$ While the number of the latter experiments is not sufficiently large to lead to a final conclusion, the data suggest that such storage has a more pronounced effect on the high molecular weight constituent present in the normal than in the infected cords

Virus Activity of Sediments from Glycerolated and Frozen Infected Tissue

The virus activities of the sediments from glycerolated and frozen infected cords were determined by finding the minimum quantity in terms of nitrogen, which would produce typical poliomyelitis when injected intracerebrally into normal *rhesus* monkeys. The results found for eleven different preparations are shown in Table IV It can be seen that with the exception of Experiment 22, infections were regularly obtained with 5×10^{-9} gm of nitrogen of the virus sediment in a volume of 1 ml., when the infected tissue had been frozen before extraction In most cases some infection was also obtained with 5×10^{-10} gm. The regular infective dose when glycerolated tissue was used was 5×10^{-8} to 5×10^{-7} gm., and only about 30 per cent of the animals tested with 5×10^{-8}

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		22	7	8	44	5	0 51	1 16
		23	85	9	103	5	0 078	0 076
		24	105	10	170	3	0 12	0 071
		26	30	12	186	3	0 19	0 10
		28	9	8	170	3	1 0	0 59
		29	13	17	73	4	0 16	0 22
		30	19	8	93	3	0 39	0 42
Normal	In 50 per cent glycerol-phosphate at 5°C	31	54	13	57	3	0 22	0 39
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		25	54	10	111	3	0 04	0 036
		32	34	4	112	3	0 18	0 16

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gm developed poliomyelitis In so far as it is possible to compare these results, therefore, the use of frozen tissue resulted in material about ten times as active as that obtained from glycerolated tissue The activity found in Experiment 22 can be explained by the fact that in this case the cords had been stored for an average of only 7 days, and the sedimentable nitrogen probably consisted of the normal component as well as of virus This explanation would also account for the relatively large yield of the high molecular substances found

TABLE IV

Activities of Individual Preparations from Glycerolated and Frozen Tissues

Experiment No.*	Method of storage	Average length of time stored	Concentration injected (gm nitrogen per ml)							
			5×10^{-6}	5×10^{-7}	5×10^{-8}	5×10^{-9}	2.5×10^{-9}	1×10^{-9}	5×10^{-10}	5×10^{-11}
		days								
6	In 50 per cent glycerol-phosphate at 5°C	345				++−−‡	−−−−			
11		26		+−	+−−	−−				
16		42	+++	++−	++−	+−−				
17		140	+++	+++	++−	+−−				
20	In stoppered tubes without solvent at −10°C	81	+++	+++	+++	+++			+−−	−−−
21		145			+	+			−	
22		7		+++	++	++−			−−−	
23		85		++	++	++	++	+−	+−	
24		105			++	++			−−	
26		30		+++	+++	++		−−−	+−−	
30		19			++	++			++	

* The experiment numbers refer to the same experiments listed in Table III In Experiment 20 the tests at 5×10^{-11} and 5×10^{-10} gm nitrogen per ml. were made when the sample was 27 days old In Experiment 21 the dilutions were made assuming a nitrogen concentration of 0 01 mg per ml. In Experiment 24, the activity tests were made after the sample had stood at about 5°C for 67 days.

‡ See footnote, Table II.

in this experiment The constituent from normal cords failed to produce symptoms of disease when doses corresponding to 5×10^{-5} gm of nitrogen were injected intracerebrally

General Properties of the High Molecular Weight Constituents from Infected and Normal Tissues

The products obtained after three or four sedimentations in the ultracentrifuge from either infected or normal tissues consisted of small, amber-colored, transparent pellets which dissolved completely in water or buffer solutions In the case of glycerolated cords in which relatively large yields of high molec-

ular weight materials were found, the solutions were in most cases opalescent by reflected light and slightly yellow by transmitted light. The products obtained from frozen cord, regardless of whether they were infected or normal, were appreciably yellow but failed to show the characteristic opalescent appearance by reflected light. The virus concentrates, in two instances, were examined for streaming birefringence by allowing the solutions to flow from a

TABLE V

Stability of Purified Virus Preparations When Stored for Different Lengths of Time in Different Solvents

Experiment No	Length of time stored	Solvent used	Concentration injected (gm. nitrogen per ml.)							
			1.5×10^{-6}	5×10^{-7}	2.5×10^{-7}	5×10^{-8}	10^{-8}	5×10^{-9}	2.5×10^{-9}	5×10^{-10}
	days									
6	1	0 05 molar phosphate at pH 7.5						++−−*	−−−−	
	26						+−−	+−−		
	51				−−	+−	+−			
	88		+++	+						
16	5	0 01 molar borate at pH 7.8	+++	++−		++−		+−−		
	21		+++−	−		+−−		−−−		
	25		+++	++−		−−−		−−		
	50		++−	+++		−−−		−−−		
20	1	Ringer's solution pH 8	+++	+++		+++		+++		
	27			+++		+++		++−		+−−
	95			++++		+++		++−		−−−

The sample obtained in Experiment 6 was prepared from glycerolated infected tissue with 0.05 molar phosphate buffer as the solvent. The samples in Experiment 16 and 20 are the same as those described in Table III. The three samples at concentrations of 0 1 2 1 and 0 15 mg nitrogen per ml. respectively were stored in stoppered tubes at about 5°C.

* See footnote, Table II.

pipette held between crossed polaroid plates In neither case was there evidence of double refraction of flow

The purified virus preparations give the Millon's test for protein. The nitrogen in one of the earlier, less active virus samples and that from a normal sample obtained in both cases from glycerolated material, was completely precipitated by trichloroacetic acid Several samples from normal and in fected cords were examined for phosphorus (10) and carbohydrate (11), and in all cases these constituents were found present. The nitrogen to phosphorus ratio for the normal constituents in the two preparations was 4.5 to 1, and for two virus samples it was 3 7 and 5.3 to 1 The carbohydrate (as glucose)

to nitrogen ratios for the same preparations were 1 1 and 1 3 for the normal samples and 1 2 and 0 3 for the virus samples However, because the analyses were carried out on extremely small samples, the experimental error was large, and the values are not greatly significant except to demonstrate that both carbohydrate and phosphorus were present

On the addition of one-half volume of saturated ammonium sulfate to solutions of either the virus-containing sediment (Experiment 30) or the normal constituent (Experiment 32), the solutions became turbid On allowing them to stand overnight in the refrigerator, a yellow amorphous precipitate separated in both cases That from the infected cord proved partly insoluble in water or in dilute phosphate buffer at pH 7 The precipitate from normal tissues dissolved completely in Ringer's solution, but its solubility was not determined in the above mentioned solvents

The purified virus is relatively stable at about 5°C when dissolved in Ringer's solution Table V shows the results of activity tests made on three preparations over a period of about 3 months Some loss of activity probably took place after the solutions had stood for about 25 days, but even after 95 days in the case of the most active preparation (Experiment 20) there was sufficient activity remaining to infect the three animals tested at a concentration of 5×10^{-8} gm of nitrogen The purified preparations do not retain their activity when dried from Ringer's solution in the frozen state and over phosphorus pentoxide at 40°C One such preparation, for example, which was 100 per cent infective at a concentration of 5×10^{-9} gm of nitrogen before it was dried, failed to infect two monkeys at $1\ 3 \times 10^{-5}$ gm after it was dried

DISCUSSION

The experiments on extracts of normal spinal cords and medullae demonstrate that there is a high molecular weight constituent present and that it is sedimented in the ultracentrifuge under the conditions used to sediment the virus Appreciable yields of this component were obtained either from glycerolated normal or infected tissues or from tissues that had been stored without glycerol at −10°C for a few days Storage of normal tissue at −10°C for longer periods of time resulted in a decrease of this constituent, and after several weeks the amount present became almost negligible Storage of infected tissue at −10°C likewise resulted in a decrease in the yield of sedimentable nitrogen, but small amounts of material were still obtained after the tissues had been stored for several months The specific activity of the sediments under the latter conditions was about ten times that of the preparations obtained either from glycerolated or fresh, infected tissues It appears, therefore, that the decrease in the yield of high molecular weight substances takes place at the expense of the normal constituent rather than of virus The experiments on the recovery of virus activity after three ultracentrifugal

cycles of purification provide evidence that the amount of virus, which had become irreversibly aggregated after ultracentrifugation, was not appreciable

The high and relatively uniform specific activity of the virus preparations obtained from cords that had been stored for several weeks suggests that they may consist of relatively pure virus. A more detailed study of the minimum period of storage necessary to eliminate the high molecular weight constituent completely from normal tissue and of the effect of storage on total virus activity is in progress. These experiments should provide additional information regarding the optimal conditions for obtaining the virus. Some direct evidence for the homogeneity of the purified preparations has been found from studies in the McBain transparent ultracentrifuge (12) In collaboration with Dr Frances Wright, the homogeneity and sedimentation rate of several preparations were determined. The results, which will be presented in full in a later paper, show that relatively homogeneous preparations were obtained in experiments in which the infected tissues had been stored for several months The average value for the sedimentation constant S_w^{20}, was 62×10^{-13} It is of interest that this value is different from that of S^{20}, $160-170 \times 10^{-13}$ reported recently for the mouse encephalomyelitis virus by Gard and Pedersen (13) If further work definitely establishes the identity of the 62×10^{-13} component with the poliomyelitis virus, it would appear that the two viruses differ not only in immunological properties as shown by Theiler (14) but in sedimentation rate as well

SUMMARY

Results of experiments on the preparation of high molecular weight constituents from normal and poliomyelitis-infected medullae-cords are presented Relatively large yields were obtained from glycerolated normal or infected tissues or from tissues that had been stored at $-10°C$ for a few days When the frozen tissues were stored for several weeks, the amount of sedimentable nitrogen isolated from the normal cords decreased and became almost negligible Under these same conditions small but definite amounts of a high molecular weight material were isolated from the infectious extracts. This material regularly produced poliomyelitis when 1 ml. containing 5×10^{-9} gm. of nitrogen was injected intracerebrally into *rhesus* monkeys. The purified virus sediment contains nitrogen, phosphorus, and carbohydrate it gives the Millon's test for protein, and is precipitated by one third saturation with ammonium sulfate Results of ultracentrifugal analyses show that a relatively homogeneous component with $S_w^{20} = 62 \times 10^{-13}$ is present.

The authors wish to thank Professor E. W Schultz for much valuable advice, for examining the histological sections and for providing many of the facilities of the Department of Bacteriology and Experimental Pathology for this work.

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ON THE MECHANISM OF SPECIFIC PRECIPITATION

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It has been pointed out (13, 10, 17) that according to the "alternation" (= "mutual multivalence" = "lattice" = "framework") theory of serological reactions, simple chemical compounds, if they contain as many as two (or at any rate three) groups capable of reacting specifically with antibody, should be able to form precipitates when mixed with the appropriate antisera. It is apparent that such precipitation would not be expected on the basis of the views of Bordet (3) concerning serological reactions, with their emphasis on the covering of the surface of the antigen by a film of antibody [1] Tests of this prediction of the alternation theory have been made (10, 11, 17) with results which are not in full agreement with the older (or the newer) theory This raises the question if either point of view can be quite correct, and it is the purpose of the present communication to report the results of experiments designed to throw light on this question.

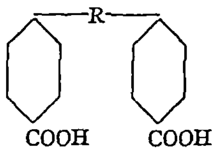
Pauling, Campbell and Pressman (17) have suggested that the failure of Hooker and Boyd (11) to obtain specific precipitation with their divalent haptens could be attributed in part to the small size of the molecules studied, stating that "Steric repulsion between two antibody molecules attached to such small molecules would be much stronger than for the molecules used by Landsteiner and van der Scheer and by us." It will be seen below that this can hardly be the true explanation, but in view of this suggestion, and in view of the fact that Hooker and Boyd failed to observe precipitation with compound "V" (see below), where R = —N=N—$C_6H_4AsO_3H_2$, but did with "VII," where R = —N=N—C_6H_4—N=N—$C_6H_4AsO_3H_2$, it was considered necessary to make compounds in which the reactive groups were about as far apart as in the latter compound.

In the course of this work 34 different compounds were made and tested Seven of these had been previously studied (11, 17), the others had not been examined, and apparently the majority of them have been made here for the first time. We may divide the parent compounds on the basis of structure into

[1] Except presumably with rather large molecules such as polysaccharides or simple molecules which associate in solution to give larger particles

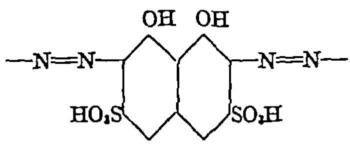
five classes, I, II, III, IVA, and IVB, which have the composition shown below, where R represents the group which varied

I

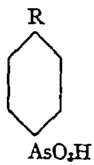


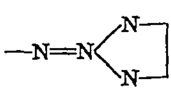
(C$_2$) R = —

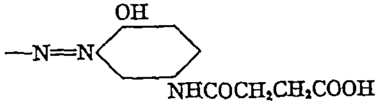
(ZC$_2$) R = —N=N—

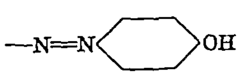
(NC$_2$) R = 

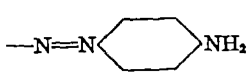
II

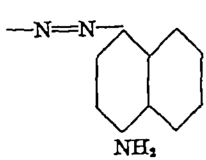


(IA) R = 

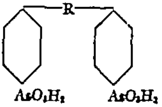
(SA) R = 

(HA) R = 

(AA) R = 

(NA) R = 

III

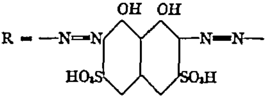


(ZA$_2$) R = —N=N—

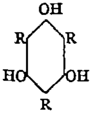
(SA$_2$) R = —NHCOCH$_2$CH$_2$CONH—

(DA$_2$)

(NA$_2$)

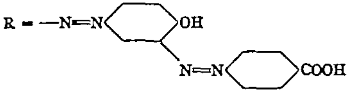


IV

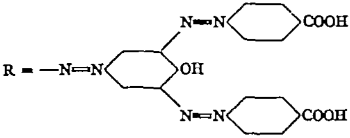


A

(PC$_3$)

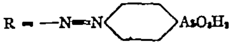


(PC$_4$)

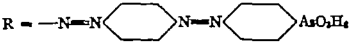


B

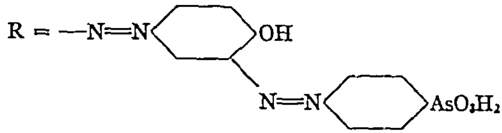
(V)



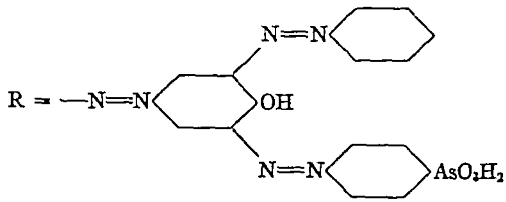
(VII)



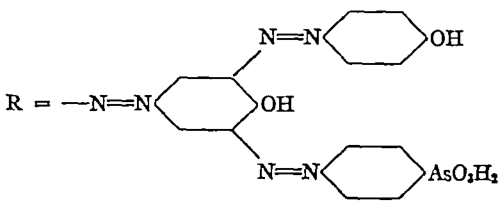
(PA_3)



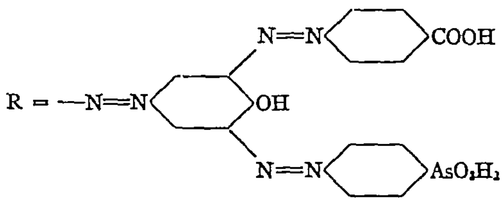
(PP_3A_3)



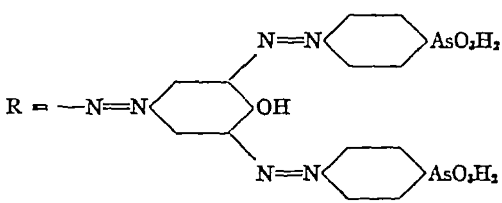
(PH_3A_3)



(PC_3A_3)



(PA_6)



The combinations of letters in parentheses are shorthand designations for the various compounds, for convenience in reference It will be noted that A represents the arsonic acid group, C the carboxylic acid group, Z the azo linkage, P phloroglucinol, etc The compounds designated by the Roman numerals V and VII are the compounds thus numbered in the paper of Pauling, Campbell, and Pressman (17)

In addition to these compounds, the following were made by acetylating or benzoylating certain of the above (a represents acetyl, b, benzoyl) NAb, NA_2b, PC_3a, PC_3b, PC_6a, PC_6b, PA_3a, PA_3b, PC_3A_3a, PC_3A_3b, PA_6a, PA_6b

All the haptens were brought into solution as the sodium salt, by addition of the minimum of NaOH The concentration of the stock solutions was in each case 1 mg /cc

Tests with a few of the haptens brought to definite pH indicated that the exact pH of the solutions was not important, doubtless because the pH of the serum hapten mixtures is in all cases controlled almost completely by the relatively large buffer power of the serum.

Materials and Methods

The preparation of compounds of class I has already been described (11) The compounds of class II were all made by coupling diazotized arsanilic acid with the appropriate substance. Of the compounds of class III NA_2 has been described (11), SA_2 was made as described by Morgan and Walton (14) DA_2 was made by the Bart reaction from

Of the compounds of class IV, V was made by coupling three equivalents of diazotized arsanilic acid with phloroglucinol (17), VII by coupling three equivalents of diazotized *p*-amino-azobenzene arsonic acid (AA) with phloroglucinol. In addition to my own preparations of V and VII small samples were available which Drs Pauling, Campbell, and Pressman kindly sent me prepared as described by them (17) no differences in behavior were observed. The other compounds were made by coupling three equivalents of diazotized *p*-amino phenol with phloroglucinol, purifying, then coupling to this in alkaline solution the appropriate amounts of other diazotized amines in the presence of pyridine (18) These compounds were purified first by repeatedly dissolving in alkali and precipitating with acid, then by crystallization from alcohol water mixtures.

The constitution of several of the arsenic-containing compounds was checked by drying and analyzing them for arsenic.

The antisera used in these experiments were of two kinds, designated as anti C and anti A. The former were made by injecting rabbits with *Limulus* hemocyanin coupled with diazotized *p*-amino-benzoic acid, the latter by injecting *Limulus* hemocyanin coupled with diazotized arsanilic acid. Four different anti C sera, each represented by two bleedings at different stages of immunization were available, *viz.*, 161, 162 163, 165 The corresponding anti A sera were 239, 241 243, 244 271 Two different bleedings from each of these rabbits were also available. The earlier bleedings seemed somewhat superior The anti C sera contained about 0 2 mg of antibody N per cc. the anti A sera about 0 3

The tests for precipitability of the various haptens were made by mixing in small tubes 0.3 cc. of antiserum with 0.3 cc. of an appropriate dilution of the hapten allowing to stand overnight in the ice box then examining for precipitate. Controls consisting of hapten alone, hapten plus saline, and hapten plus normal rabbit serum were always included, and were consistently negative.

RESULTS

None of these haptens were precipitated by any of the anti-C sera, in any of a large number of dilutions tested The results with compounds of classes I and IVA were thus consistently negative

None of the univalent arsenic-containing haptens (class II) were precipitated It will therefore be unnecessary to present details of tests with compounds of any of these three classes

TABLE I

Tests for Specific Precipitation of Anti-A Sera Mixed with Haptens of Class III

Serum No	Hapten	Initial concentration of hapten (micrograms per cc.)				
		1000	200	40	8	1 6
271-I	ZA_2	–	–	++	+	–
239-I	ZA_2	–	+w	++	+	–
271-I	SA_2	–	–	–	–	–
239-I	SA_2	–	–	–	–	–
271-I	DA_2	–	–	–	–	–
239 I	DA_2	–	–	–	–	–

– means no precipitation, +w means very slight precipitation, + means definite precipitation, ++ means marked precipitate formation

TABLE II

Tests for Specific Precipitation of Anti-A Sera Mixed with Haptens of Class IV B

Serum No	Hapten	Initial concentration of hapten (micrograms per cc.)				
		1000	200	40	8	1 6
243-I	PA_3	–	–	–	–	–
271-I	PA_3	–	–	–	–	–
239-I	PC_3A_3	–	–	–	–	–
271-I	PC_3A_3	–	–	–	–	–
271-I	PH_3A_3	–	–	±	–	–
271-I	PP_3A_3	–	±	+±	+w	–
243-I	PA_6	–	–	–	±	–
271-I	PA_6	–	–	–	+	–

± means doubtful formation of precipitate. Other symbols as in Table I.

The results of tests with compounds of class III are shown in Table I It may be seen that only one (ZA_2) of these divalent haptens gave any precipitate

Of compounds of class IVB, a number were specifically precipitable by anti-A sera, in a way which was seen to depend definitely on their constitution, but not in the way demanded by the "alternation" theory

The results obtained with compounds of class IVB are shown in Table II. (It will be recalled that class IVA (carboxy compounds) was entirely negative) Of the haptens shown in Table II, two were negative, one gave a very weak reaction, another a slight positive reaction, and only one precipitated well. All the compounds which failed to precipitate were retested with other anti-A sera, with the same results, and compounds which did precipitate were tested with other anti A sera and with unrelated antisera to control the specificity of the reactions. These results are not shown here.

DISCUSSION

It is apparent from Tables I and II, and the results obtained with compounds of classes I and IVA, that predictions based on the alternation theory are by no means regularly fulfilled No carboxy compound was ever observed to precipitate, and many of the arsonic haptens, even those containing three reactive groups, adequately separated, failed to precipitate In all, six divalent haptens, four trivalent, and one hexavalent, failed to precipitate. These compounds nevertheless reacted with the appropriate antibodies, as was shown by inhibition experiments. It seems clear that the possibility of "lattice" (framework) formation is by no means sufficient to insure that a hapten will precipitate. As a matter of fact, a careful examination of Table II discloses a different sort of correlation between constitution and precipitability which probably provides a much more valid basis on which to predict the behavior of haptens.

It may be assumed that all the arsonic acid groups in the first four haptens shown in Table II are capable of combining with molecules of antibody, and calculations of the size of antibody molecules and the relative distances in these hapten molecules support this idea This combination blocks off the solubilizing action which the arsonic acid groups are known to exert, and at the same time at least one, possibly several, polar groups of the antibody are combined with The result is a complex of one hapten and three antibody molecules, which being larger than one antibody molecule, demands at least three times, perhaps more, the number of solubilizing polar groups which would just suffice to keep an antibody molecule in solution. From what is known of protein chemistry, we may surmise that this latter number probably lies somewhere between 5 and 40 In hapten PA_3, three outer[2] hydroxyl groups still remain uncombined with, and in PC_3A_3 there are in addition three carboxyl groups This, in addition to the polar groups remaining free on the antibody molecules, evidently is sufficient to keep the complex in solution, for no precipitate is formed The importance of solubilizing groups in immune reactions has been commented on by Eagle (6) who observed that the introduction of a

[2] The inner hydroxyls, *i.e.*, those on the phloroglucinol residue, are probably too completely blocked off mechanically (by steric hindrance) to have their full solubilizing effect.

few soluble groups into the antitoxin molecule rendered it incapable of flocculating toxin, although it could still combine

If the size of the hapten, and at the same time its non-polar character, are increased by the addition of three phenyl groups, as in hapten PP_3A_3, the result is so insoluble that the three free hydroxyl groups are no longer sufficient, and the compound is precipitable If the size is similarly increased, but the polar character kept approximately the same by the introduction of three new hydroxyl groups, as in hapten PH_3A_3, the resulting hapten does not precipitate, or precipitates only slightly

From spatial considerations, it is doubtful if all six of the reactive groups in hapten PA_6 can simultaneously combine with antibody molecules, but it is

TABLE III

Tests for Specific Precipitation of Acetylated (a) and Benzoylated (b) Derivatives of Compounds of Class IV B

Serum No	Hapten	Initial concentration of hapten (micrograms per cc.)				
		1000	200	40	8	1 6
271-I	PA_3a	−?	+	±	−	−
271-I	PA_3b	−	−	±	+	−
271-I	PC_3A_3a	−	−	+w	−	−
271-II	PC_3A_3a	−	−	+w	−	−
271-I	PC_3A_3b	−	−	−	±	−
271-I	PA_6a	−	−	++	+	±
271-I	PA_6b	−	−	+w	++	+

Symbols as in Tables I and II

probable that more than three are capable of doing so, leaving less than three solubilizing groups in addition to the hydroxyls It is therefore not surprising that this compound precipitates, although it will be noted that its precipitability is much inferior to that of PP_3A_3 (or of ZA_2 or VII) It is probable that a mechanism proposed by Hooker and Boyd (11), namely, mechanical occlusion of polar solubilizing groups of neighboring antibody molecules, also comes into play in this instance, and perhaps in the above cases too

If the above explanation were correct, we should expect that acetylating or benzoylating the unprecipitable class IV haptens, so as to block off the solubilizing hydroxyls, would render them specifically precipitable That this is so is shown by Table III, which gives the results of tests on acetylated and benzoylated derivatives of some of the compounds of Table II

It is of considerable interest that the precipitability of the acetylated or benzoylated PC_3A_3 is very slight, which may doubtless be attributed to the

three carboxyl groups still remaining In fact the precipitability of PC_3A_3a and PC_3A_3b is quite comparable with that of the unmodified PA_4 (Table II) Similarly benzoylation of hapten NA_2, which in addition to the two hydroxyls has two sulfonic acid groups, did not render it precipitable.

The relation of structure to precipitability in these compounds seems quite clear, and the possibility of "lattice" formation is evidently quite irrelevant, even if we grant the unproven assumption that the anti-A antibody is divalent How may we explain the results obtained with divalent haptens of class III, shown in Table I, where one compound precipitated, whereas two did not, although few if any additional solubilizing groups are present?

It seems probable that the explanation of these results again rests upon the mechanical occlusion of polar groups of the neighboring antibody molecules, which in hapten ZA_2 would be brought quite close together, whereas in haptens SA_2 and DA_2 they would be farther separated, so that all their polar groups, with the exception of those actually concerned in combining with the arsonic acid groups, would remain free to keep the complex in solution. If this suggestion is correct, the idea of Pauling, Campbell, and Pressman, that the smaller hapten molecules cannot precipitate, is erroneous Instead, the converse would seem to be true, namely, the reason haptens SA_2 and DA_2 do not precipitate is that they are too large (*i.e.*, their combining groups are too widely separated)

The above interpretation is strengthened by the observation that univalent haptens (class II) never precipitate, even in the case of a biggish molecule (NAb) having no free polar groups in addition to the arsonic acid group through which combination is effected Evidently the reduction in free polar groups of the antibody molecule which follws this combination is by itself insufficient to reduce the solubility significantly, in the absence of mechanical hindrance due to the near by presence of another molecule of antibody

It is not easy to say precisely how these considerations apply to the precipitation of the divalent haptens studied by Landsteiner and van der Scheer (12), such as resorcinol-disazo-*p*-suberanilic acid At first sight, it might seem that in this hapten the two reactive groups are quite separated. However, the antisera used in these experiments were rather specific for the various anilic acids made from fatty acids of various lengths, which may indicate that the antibody when reacting with this hapten combined not only with the carboxy group but with the whole side chain right up to the resorcinol residue, which would bring the two molecules of antibody in rather close apposition. Only two solubilizing groups, the two hydroxyls of the resorcinol, then remain, and they may be somewhat hindered by the presence of the antibody molecules. Our present knowledge of the numbers of polar groups required to keep such complexes in solution does not seem sufficient to enable us to say whether this would account for the precipitability That it may is suggested by the ex-

planation offered by Landsteiner and van der Scheer themselves, which was in terms of peculiarities in constitution such as the long aliphatic chains, which would be fairly insoluble The observation that these compounds precipitated better after their solutions had been allowed to stand, however, unlike the compounds studied by Pauling, Campbell, and Pressman, and by myself, suggests, as Landsteiner and van der Scheer pointed out, the possibility that this hapten might be somewhat aggregated in solution, giving particles possessing several combining groups, and large enough to combine simultaneously with several molecules of antibody

The failure of any of the carboxy compounds to precipitate with anti-C sera rests undoubtedly upon the poor flocculating quality of these sera Although they gave good reactions by the interfacial ("ring") technique, these sera, even when fresh, flocculated only slowly with casein coupled with diazotized *p*-aminobenzoic acid (I have previously observed that the carboxy group does not have nearly the antigenic power of the arsonic acid group) The precipitation of the arsenic-containing haptens was observed to be relatively slow compared to the flocculation of casein-arsanilic compounds by the same sera, in which case it was practically instantaneous It is therefore not surprising that the much less "avid" anti-C antibodies could not effect precipitation of the carboxy haptens, all of which would be much less precipitable than conjugated protein antigens Failure to take account of such facts seems to be another way in which the alternation theory is an oversimplification of the true mechanism [3]

Finally we must discuss the failure of compound V to precipitate, when hapten VII, apparently so similar, precipitated readily We can hardly avoid mention of this fact on the basis of the report of Pauling, Campbell, and Pressman that V did precipitate, for the discrepancy may possibly depend upon the differences in the antibodies in their sera and in mine, in which case it is unknown how the compounds studied here would have reacted with their sera There is no doubt that V did not precipitate in my hands, it was tested against both bleedings of each of the five anti-A sera prepared, while these sera were still fresh and capable of precipitating hapten VII powerfully In not a single one of these mixtures, no matter what the concentration of V, was the faintest trace of precipitation or clouding observed

It seems likely that the failure of V to precipitate is due to the fact that the

[3] The test of the theory carried out by Hooker and Boyd (11) therefore seems to have been a fair test, within the framework of the hypothesis itself It is rather interesting to consider, however, that if their sera had been sufficiently "avid," comparable to the anti-A sera studied here (assuming such anti-C sera can be made), Hooker and Boyd might have observed precipitate formation with the shorter of their haptens Since the interpretation which they would have put on this result would probably have been the erroneous one that the alternation theory is perfectly correct, it is perhaps fortunate that this did not happen

combining groups are insufficiently separated for more than two molecules of antibody to be able to combine simultaneously with the molecule, so that there are always free three hydroxyls and one arsonic acid group, which are sufficient to keep the complex soluble. In addition, the polar groups of the antibody molecules not concerned in the combination, might remain relatively free, as the antibody molecules would not be forced into intimate enough contact. In VII, the combining groups, being more widely separated, are probably all three able to combine simultaneously with antibody molecules. This leaves no arsonic acid groups free, and probably results in a good deal of steric hindrance of polar groups on the antibody molecules

It is thus seen that the *possibility* of "framework" formation is by no means sufficient to insure specific precipitation, for although this possibility does not exist with hapten V if the above interpretation of the behavior of V is correct, it does exist in the case of haptens PA_3 and PC_3A_3 which did not precipitate, and hapten PH_3A_3, which gave only a trace of precipitate. Conversely, the possibility of framework formation does not seem to be necessary for precipitation, for hapten ZA_2 precipitated readily, which it could not do by framework formation unless the valence of antibody is more than two, which there is some reason to doubt, both on experimental grounds, and on the basis of theories of antibody formation (15, 2, 5, 16) It is true that Pauling has described a possible mechanism for the formation of trivalent antibody, but this seems to the present author to have been introduced chiefly for the purpose of accounting for the precipitation of divalent haptens, and to have no great plausibility, even from the point of view of Pauling's own theory There is no experimental evidence for it

In considering the bearing of these experiments on the "alternation" theory, we should keep clearly separted in our minds two aspects of this theory The first is the clearly implied claim of its proponents that the possibility of framework ("lattice") formation is necessary and sufficient for the initiation of a serological reaction ("   aggregation would occur regardless of the affinity of the groupings for water" (7)) The second is the claim that larger aggregates are formed solely by the specific linkage of antibody groups with antigen groups It seems to the present author that the experiments reported here completely disprove the first claim of the alternation theory On the other hand, it is clear that if antibody is always divalent, these experiments do not bear particularly on the second claim.[4] I do not wish to be understood as denying that the alternation theory, in making this second claim, may be entirely justified Indeed, evidence indicating that in certain cases the formation of larger aggregates is a phenomenon of a certain degree of specificity (19, 20) is difficult to explain, on the basis of present knowledge, unless we assume some such mechanism.

[4] The precipitability of the divalent hapten ZA_2 would appear to demand trivalent antibody

The question would seem to depend ultimately on whether antibody molecules have in general more than one combining group, and the evidence now available is not sufficient to settle this point

As an explanation of the *cause* of precipitation, it would seem that neither the alternation nor the Bordet theory is adequate The alternation theory seems to be simply incorrect, and the Bordet theory too vague to account for the very definite facts presented here For the theory suggested above (which is probably not original with me[5]), namely, that precipitation is due to lowering of solubility by neutralization of polar groups of antibody and hapten (or antigen) and concommitant steric hindrance of other polar groups of neighboring antibody molecules in the complex, I wish to propose the name, occlusion theory

It will be noted that the occlusion theory simply attempts to explain why combination of antibody and antigen or hapten produces, in certain cases, a compound having too low a solubility to remain in solution Nothing is said about the mechanism by which these primary aggregates unite with each other to build up the larger aggregates which are observed to form during serological precipitation I shall for the present make no attempt to provide a detailed hypothetical mechanism for this, any more than I propose to explain how the aggregates are formed which result when a protein is salted out of solution by ammonium sulfate *If* antibody has more than one combining group, it may well be that in specific precipitation the primary aggregates unite in a way very similar to that demanded by the alternation theory, indeed, it is hard to see how such unions could fail to play a prominent rôle in the formation of larger aggregates It must be remembered, however, that "apparently decisive evidence" (8) has been offered that under some circumstances aggregates can be built up by a non-specific mechanism not involving "framework" formation (1, 9, 4)

If antibody were trivalent, it would certainly seem that all the divalent and trivalent haptens studied here should have been able to form frameworks, and this would be true with the trivalent haptens even if antibody were only divalent Also divalent antibody ought to be able to build up with divalent haptens long chain-like aggregates showing pronounced birefringence of flow (Pauling), but this has not been observed (11) Until more experimental evidence is available, the multivalency (including divalency) of antibody remains almost purely a postulate In any case, the present communication attempts simply to present evidence which seems to throw light on the reason for the tendency of primary aggregates not to remain in solution, a reason which seems to have been overlooked by the proponents of the alternation theory

SUMMARY

A study of the precipitability by the appropriate antisera of 34 different haptens, containing from one to six reactive groups, leads to the conclusion that

[5] Compare Marrack (13), p 150

the possibility of framework ("lattice") formation is neither necessary nor sufficient for specific precipitation, but that instead precipitation depends upon the reduction, by mutual neutralization of polar groups of antibody and antigen (or hapten) and mechanical blocking off of polar groups of closely neighboring molecules of antibody, of the solubility of the complex below the point at which it can remain in solution The decisive factors appear to be the number of polar groups of the antigen (hapten) left free, and the distance separating the different reactive groups, which determines the amount of steric hindrance exerted by one antibody molecule on another No hypothesis is offered as to how these primary insoluble aggregates unite with each other to produce the larger aggregates which are finally observed

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THE RÔLE OF THE COMPONENTS OF COMPLEMENT IN SPECIFIC IMMUNE FIXATION*

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Although it is known that complement combines with most antigen antibody compounds, the rôle of the components of complement in this union is not clearly understood

It is generally stated that the mid piece of complement is the combining component (1, 2) On the other hand, Brin states that the end-piece alone is fixed (3) Others assert that both the end piece and mid piece combine with antigen antibody compounds (4–7) Deissler (8) contends that the fourth component of complement is utilized in complement fixation Some workers claim to have confirmed this statement (9, 10), while others contradict it (11)

The present authors, in experiments preliminary to those reported in this paper, found indications that the fourth component and end piece, as well as mid piece in a smaller proportion, combine with antigen-antibody compounds

There are several reasons for the various views of others. (1) With the exception of a few recent studies, investigations on the rôle of the complement components in fixation were performed before the discovery of the fourth component of complement. (2) Uniform methods for the preparation and identification of the individual complement components were not employed. (3) Much of the past work was qualitative in nature with little consideration of the physical and chemical conditions which affect the combination of complement with antigen-antibody aggregates. (4) The results obtained under one set of experimental conditions were frequently interpreted to fit all other conditions.

The need for more rigidly controlled studies on complement fixation is evident, and accordingly the present paper is concerned first with the conditions governing the fixation of the components of complement, and second with the identification of those components of complement which combine with specific antigen-antibody compounds.

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Résumé of the Nature of the Components of Complement

To assist in the interpretation of the data presented in this paper, a review of the existing knowledge of the constitution of complement is necessary A more detailed discussion has been presented elsewhere (12)

Complement can be separated into two thermolabile components (destroyed by heating at 56° for 30 minutes) by treatment of serum with carbon dioxide-saturated water or dilute HCl, or by dialysis against distilled water (1) Accompanying each of these fractions are varying amounts of two relatively thermostabile components of complement, the third and fourth components The fraction of fresh serum insoluble in a tenfold dilution of carbon dioxide-saturated water has been termed the "globulin fraction" or the "mid-piece," while the fraction soluble in the same medium has been termed the "albumin fraction" or "end-piece " The terms mid- and end-piece were formulated by the early investigators because they supposed that the carbon dioxide-insoluble fraction had first to combine with the sensitized red blood cells before the albumin fraction would act. It is also known that either cobra venom or yeast cells destroys a relatively heat-stable component (inactivated by heating at 66°C for 30 minutes) which has been termed the third component (1) It is usually associated with the globulin fraction of serum, although, as will be shown below, it is also nearly always present in the end-piece Pillemer and Ecker (13), investigating the nature of the yeast inactivation of the third component, isolated an insoluble fraction from yeast which adsorbs the third component specifically from fresh serum. Certain unpublished data are indicative of a lipid nature of this component (14) A fourth component of complement, inactivated by dilute ammonia, has also been discovered (1) It is usually associated with the end-piece, but, as will be shown below, under certain experimental conditions it also is often present in the mid-piece fraction of complement Pillemer, Seifter, and Ecker (15) postulated that this component contains a reactive carbonyl group and is associated with a globulin fraction present in the so called albumin fraction

Pillemer, Ecker, Oncley, and Cohn (16) have prepared in a highly pure state, and characterized, three of the components of complement The mid-piece was found to be a euglobulin with an apparent isoelectric point of about 5 2 It has an electrophoretic mobility of 2 9 $\times$ 10^{-5} in a phosphate buffer of an ionic strength of 0 20 at pH 7 7, and a sedimentation constant of 6 4 $\times$ 10^{-13} in KCl of ionic strength of 0 20 It comprises only 0 60 per cent of the total serum protein, and each cubic centimeter of serum contains 0 060 mg of mid-piece nitrogen The end-piece and fourth component were found in the same serum fraction, which was characterized as a muco-euglobulin with an apparent isoelectric point of 6 3–6 4, and with an electrophoretic mobility of 4 2 $\times$ 10^{-5} It contains 10 3 per cent carbohydrate, and it comprises less than 0 2 per cent of the total serum protein, thus accounting for 0 02 mg of nitrogen per cc. of serum.

Pillemer and Ecker (17) on the basis of electrophoretic and functional studies, and Heidelberger (18, 22) have proposed a new nomenclature for the complement components The symbols C′1, C′2, C′3, and C′4, which correspond to the mid-piece, end-piece, third component, and fourth component respectively in the older termi-

nology, were adopted. In the present paper the terms mid-piece and CO_2-insoluble fraction are used interchangeably as are end-piece and CO_2-soluble fraction, and the symbols C'1 and C'2 represent the mid piece and end-piece *components per se*. The terms third and fourth components are used interchangeably with the symbols C'3 and C'4 respectively.

Materials

Complement.—The blood of normal guinea pigs which had been maintained on a high vitamin C diet was placed in the ice box for about one hour. At the end of this time the serum was separated by centrifugation and immediately used in the experiments. In the protocols which follow the serum, unless otherwise noted, was never allowed to stand more than one hour before use in fixation experiments.

Antigens.—Type III pneumococcus specific carbohydrate was used in the larger part of the experimental work. A stock solution of 0.1 per cent SIII[1] in 0.9 per cent saline was diluted with 0.9 per cent saline to the desired concentration.

Human serum, hemocyanin (from *Busycon canaliculata*), tobacco mosaic virus and red blood cells of various species were also employed as antigens.[2] The amounts employed are indicated in the protocols.

Antisera.—A refined anti Type III pneumococcus rabbit serum containing 4.57 mg. of antibody nitrogen per cc. was kindly furnished by the Lederle Laboratories, Inc. It was not anticomplementary in twice the quantities employed in the present experiments.

Antihuman rabbit serum, antihemocyanin rabbit serum, antitobacco mosaic virus (rabbit) serum, and rabbit antisera to red blood cells of sheep, dog, guinea pig and man were also employed. Since the latter sera were found to be somewhat anti-complementary in the amounts necessary for optimum fixation, the procedure advocated by Sachs (19) was employed to remove the objectionable substances from them. This was successfully accomplished without much loss of the fixative powers of the antisera.

Chemicals.—All chemicals used were either best grade Eastman or Merck's blue label chemicals. Unless otherwise noted, all dilutions were made with 0.9 per cent NaCl. The insoluble carbohydrate from yeast used in the removal of C'3 was prepared by the method of Pillemer and Ecker (13).

Methods

General Method for Fixation.—To A cc. of antiserum, B cc. of undiluted fresh serum were added with thorough mixing. The mixture was then brought to the desired temperature, tonicity and hydrogen ion concentration. C cc. of antigen, adjusted to the desired experimental conditions, were then added rapidly with constant stirring.

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[1] S with the appropriate numeral is used for the type-specific polysaccharide of the pneumococcus.

[2] We are indebted to Dr. M. Heidelberger for a generous supply of anti Type III pneumococcus rabbit serum, to Dr. S. B. Hooker for hemocyanin and antihemocyanin rabbit serum, and to Dr. W. M. Stanley for crystalline tobacco mosaic virus.

The mixture was allowed to stand for the prescribed time at the required temperature Following this incubation the mixture was centrifuged for 30 minutes at 2750 R.P.M., and the clear supernatant was drawn off carefully with a pipette, adjusted to the required conditions, and finally made up with 0 9 per cent NaCl to a 10 per cent dilution of the original guinea pig serum This solution was tested for hemolytic activity, and if found not to show more than 10 per cent of its original activity it was further tested for content of individual complement components

Complement Titration —A unit of complement, designated as producing 100 per cent hemolysis, was considered to be the smallest amount of a 10 per cent dilution of untreated serum which caused complete hemolysis of 1 cc of 2 5 per cent suspension of sheep red blood corpuscles containing 5 units of antisheep cell rabbit serum per cc. An identical amount was employed in all reactivations and complement component determinations

Estimation of the Individual Component Activity in Fixed Complements —A unit of the complement rendered inactive by fixation was added to an equal amount of each of the specifically inactivated complement components described below The mixture was incubated at room temperature for 15 minutes, 1 cc of sensitized sheep red cell suspension added, and the hemolytic titer read after 30 minutes of incubation at 37 5°C The amount of hemolysis produced was estimated by comparison with a series of standard hemoglobin solutions, and expressed as percentage of complete hemolysis

It is pointed out that the data presented in this paper do not indicate the absolute quantity of each component present, but only represent the residual activity present after fixation or specific inactivation

For the further clarification of the data, the per cent hemolysis resulting from the inter-reactivations of the "specifically inactivated complements" (see below) is included in each protocol.

Success in the reactivations depends largely on preventing the loss of activity of the complement components which might occur during the course of an experiment Therefore, all serum reagents should be kept at 0°C (±1°) until they are to be used. Precautions of this kind are necessary for obtaining comparable, reproducible data Furthermore, slight deviations from any technique described here may alter the end-result of an experiment

In the interpretation of data on hemolysis no significance is attached to differences of 10 per cent hemolysis or less, because of the proscribed limit of accuracy of the method.

The Preparation of Complements Deprived of Various Components

These are hereafter designated "specifically inactivated complements"

1 Complement was deprived of active C′4 by appropriate treatment of serum with hydrazine or ammonia (15, 20)

2 Complement was deprived of C′3 by treatment of serum with zymin or the insoluble carbohydrate prepared from fresh yeast (13)

3 Complement was deprived of C′1 and C′2 activity by heating serum at 56°C for 30 minutes

4. The CO_2-soluble and CO_2-insoluble fractions were prepared by saturation of diluted serum with CO_2 as described elsewhere (12) While this method results in the sharp separation of C'1 and C'2 from one another, marked variation occurs in the distribution of C'3 and C'4 in the fractions. The CO_2-soluble fraction or end piece, contains all of the active C'2, usually from 70 to 100 per cent of the C'4, and from 20 to 40 per cent of the C'3 The CO_2-insoluble fraction, or mid-piece contains all of the C'1, usually about 60 to 80 per cent of C'3, and from 0 to 30 per cent of the C'4 In this laboratory it is believed that the residual C'4 manifest in the mid piece is accompanied by a small amount of C'2, since it is the opinion here that C'2 and C'4 are associated in the same guinea pig serum protein constituent.

EXPERIMENTAL

Factors Which Influence the Fixation of the Components of Complement—In these experiments complement fixation was carried out with anti Type III pneumococcus rabbit serum compounds

1 The Effect of Aging of Complement—

A pool of fresh guinea pig serum was divided into two portions, one of which was allowed to incubate for one hour with the specific antipneumococcus complexes, at the end of which time the fixability of the complement components was determined. The second portion of serum was allowed to stand for 19 hours in the ice box (5 C.) and was tested in a like manner The serum lost 50 per cent of its complementary activity during the 19 hours in the cold. Complete hemolysis was brought about by 0 07 cc. of 10 per cent dilution of fresh serum while 0 14 cc. of 10 per cent dilution of aged serum was required.

The results of this experiment, performed in quintuplicate, are tabulated in Table I An inspection of the data shows that when serum stood for 19 hours and lost 50 per cent of its complementary activity the following changes in the fixability of the complement components occurred (*a*) C'4 became more resistant to fixation, (*b*) C'3 underwent no change in fixability, (*c*) there was less reactivation upon the addition of the CO_2-insoluble fraction, and increased reactivation upon the addition of the CO_2-soluble fraction in the case of the aged serum

Most investigators have allowed complement to age or "cure" in an ice chest overnight before using it in fixation experiments (18, 21), however, it is apparent from the data presented here that this procedure may lead to serious error in the determination of the fixability of the various components of complement. In this laboratory, therefore, it is the practice to allow serum complement to stand not more than one hour before use in fixation experiments

2 The Effect of Time of Incubation—An experiment was performed to determine the optimum time necessary for the fixation of the various components of complement.

The reagents were mixed as described in the section of this paper dealing with the general method of fixation, and were allowed to stand at 25°C for different periods of time At the end of the allotted time the residual complement component activities were determined. In order to avoid occlusions and uneven distribution of reagents, separate tubes were employed for each incubation

TABLE I

The Effect of Aging on the Fixation of Complement

1 8 pneumococcic antiserum	Guinea pig serum	1 40 000 SIII	Hemolysis produced by supernatant after fixation		Hemolysis produced after addition of specifically inactivated complements: Serum treated with hydrazine		Serum treated with insoluble carbohydrate		Serum heated at 56 C for 30 mins.		CO_2-insoluble fraction ("mid piece")		CO_2-soluble fraction ("end piece")	
			I*	II‡	I	II	I	II	I	II	I	II	I	II
cc	*cc*	*cc*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0 2	1	0 2	0	0	0	25	50	55	10	5	65	40	20	35
0 2	1	0 2	0	0	0	25	60	60	Tr	5	65	40	15	30
0 2	1	0 2	0	0	0	25	50	60	10	Tr	65	40	15	25
0 2	1	0 2	0	0	0	25	50	60	10	Tr	65	40	15	25
0 2	1	0 2	0	0	0	30	60	50	15	Tr	65	45	15	30
Specifically inactivated complements			Hemolysis I	Hemolysis II										
			per cent	*per cent*										
Serum treated with hydrazine			0	0	—	—	80	60	80	90	40	60	90	90
Serum treated with insoluble carbohydrate			0	0	80	60	—	—	60	35	60	60	40	25
Serum heated at 56°C for 30 mins.			0	0	80	90	60	35	—	—	0	0	0	0
"End-piece"			0	0	90	90	40	25	0	0	85	90	—	—
"Mid-piece"			0	0	60	40	60	50	0	0	—	—	85	90

* I, fresh sera.

‡ II, sera after standing 19 hours at 5°C

The results of this experiment show that C′4 was fixed in the first 5 minutes of incubation and remained fixed for the duration of the 80 minute incubation Maximum fixation of mid-piece occurred after 40 minutes of incubation, while little or no variation in the fixation of end-piece occurred that could be attributed to the effect of time of incubation On the basis of these data an incubation time of 60 minutes was chosen as the optimum time to be used in the remaining fixation experiments

3 *The Effect of Temperature*—Although earlier workers (23, 24) determined the optimum temperature for the fixation of complement (considered as a single entity), no knowledge exists concerning the influence of temperature on the fixation of the individual components of complement. The results of an experiment performed to determine the effect of temperature on the fixation of the individual components reveal that maximum fixation of all the components occurred at 22°C Although slight differences in the fixability of the components were evident at 0° and 22°, marked differences occurred as between these temperatures and 37°C At the latter temperature C'4 was more resistant to fixation. On the basis of these data further fixation experiments were conducted at temperatures between 20 and 25°C

TABLE II

The Effect of Varying Hydrogen Ion Concentration on the Fixation of Complement

pH*	Relative quantity and appearance of precipitate	Hemolysis produced by supernatant after fixation	Hemolysis produced after addition of specifically inactivated complements: Serum treated with hydrazine	Serum treated with zymin	Serum heated at 56°C. for 30 mins.	CO_2-soluble fraction ("end-piece")	CO_2-insoluble fraction ("mid-piece")
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
5 3	++++ plaque	0	15	15	20	50	0
6 1	+++ plaque	0	15	20	20	45	0
7 0	++ granular	0	0	60	0	35	0
7 1	++ granular	0	0	40	0	15	0
8 1	++ granular	0	0	50	0	25	0
8 8	++ plaque	0	0	50	0	25	0

Specifically inactivated complements	Hemolysis	Serum treated with hydrazine	Serum treated with zymin	Serum heated at 56°C. for 30 mins.	CO_2-soluble fraction ("end-piece")	CO_2-insoluble fraction ("mid-piece")
	per cent					
Serum treated with hydrazine.	0	—	70	90	90	0
Serum treated with zymin.	0	70	—	80	25	60
Serum heated at 56 C. for 30 mins.	0	90	80	—	0	0
"End piece	0	90	25	0	—	85
"Mid-piece'	0	0	60	0	85	—

* 1 cc. of guinea pig serum was fixed by the aggregate from 0.2 cc. of 1 4 antipneumococcus rabbit serum and 0.2 cc. of 1 10 000 SIII All reagents were made up to volume with phosphate buffers of ionic strength 0 15 The pH indicated here is the pH of the supernatants after fixation.

4 *The Effect of Hydrogen Ion Concentration*—The results of an experiment to determine the effect of hydrogen ion concentration on the fixation of the individual complement components are shown in Table II.

In this experiment all reagents were either diluted or made up to volume with phosphate buffers of various hydrogen ion concentrations and of ionic strength 0 15 The complement was incubated with the antigen-antibody complexes for one hour at a temperature of 23°C Before determination of their complement component activities the supernatants were adjusted to a neutral pH with 0 1 N HCl or 0 1 N NaOH

Table II shows the following (*a*) at pH's below 7 0, less fixation of C'4 and C'1 occurred than at pH 7 0, but there was an enhancement of the fixation of C'3, (*b*) at pH's above 7 0 up to 8 8 there occurred complete fixation of C'4 together with an increased fixation of C'1, and (*c*) optimum fixation of the components of complement occurred at neutral pH's

In this experiment the CO_2-insoluble fraction contained no C'4, and no reactivation resulted upon the addition of CO_2-insoluble fraction to complement rendered inactive by fixation This demonstrates that C'2 was bound along with C'4 If, however, C'4 is present in the CO_2-insoluble fraction, reactivation by the latter of fixed sera in which no C'4 and C'2 can be demonstrated, is due to the presence of small amounts of C'2 in the CO_2-insoluble fraction

Another point of interest evident from the experiment is that complementary activity was removed by specific aggregates over an extremely wide pH range, although there was marked variation in the fixation of the individual components at different pH's On the basis of these data all other fixation experiments were conducted at pH's between 7 0–7 2

5 *The Effect of Tonicity*—Although the effect of varying salt concentration on the fixation of complement is well known (21), nothing is known of the effect of variations of tonicity on the fixation of the individual complement components The results of an experiment performed to determine this effect revealed the following (*a*) complement in slightly hypertonic NaCl solutions was fixed incompletely, and (*b*) complement in hypotonic NaCl solutions was fixed completely with little or no variation in the fixation of the individual components

$CaCl_2$ is known to interfere with complementary activity (1), and is further known to interfere with certain adsorption reactions (25) An experiment performed to show the effect of $CaCl_2$ on the fixation of the components revealed the following points (*a*) in amounts over 0 2 per cent, $CaCl_2$ interfered with the fixation of C'4 and C'1, and (b) in all amounts used $CaCl_2$ did not influence the fixation of C'3

6 *The Effect of Propionaldehyde*—Pillemer, Seifter, and Ecker (15) have shown that propionaldehyde protects the fourth component from inactivation by hydrazine Since the direct and immediate inactivation of complement by specific aggregates appears to involve C'4, experiments were performed to determine if propionaldehyde would interfere with the fixation of the components The results of such an experiment are given in Table III It is seen

that propionaldehyde in amounts far greater than that needed to oppose the anti C′4 effect of hydrazine failed to inhibit the fixation of complement. However, slightly less C′4 was fixed in the presence of propionaldehyde, while there was no interference with the fixation of the other components

7 *The Effect of Dilution of Complement*.—Table IV summarizes the results of an experiment undertaken to determine the fixability of the components of complement which had been diluted previous to fixation. The table shows

TABLE III

The Effect of Propionaldehyde on the Fixation of Complement

1:8 pneumococcic antiserum	Guinea pig serum	0.16 molar propional dehyde	0.9 per cent saline	1:40 000 SIII	Hemolysis produced by supernatant	Hemolysis produced by supernatants after addition of specifically inactivated complements: Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum heated at 56°C. for 30 mins.	CO₂-soluble fraction ("end-piece")	CO₂-insoluble fraction ("mid-piece")
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.2	1	1	0	0.2	0	10	50	10	15	55
0.2	1	0.5	0.5	0.2	0	10	50	10	10	60
0.2	1	0.25	0.75	0.2	0	5	60	10	15	70
0.2	1	0.125	0.87	0.2	0	10	60	Tr	10	65
0.2	1	0	1	0.2	0	0	60	Tr	10	65

Specifically inactivated complements	Hemolysis	Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum heated at 56°C. for 30 mins.	CO₂-soluble fraction ("end-piece")	CO₂-insoluble fraction ("mid-piece")
	per cent					
Serum treated with hydrazine....	0	—	80	80	90	40
Serum treated with insoluble carbohydrate	0	80	—	60	40	60
Serum heated at 56 C. for 30 mins.	0	80	60	—	0	0
"End-piece"	0	90	40	0	—	85
"Mid-piece"	0	60	60	0	85	—

that (*a*) the fixation of C′4 was inhibited only in very large dilution, and (*b*) there was an increase of fixation of the mid-piece and end piece on dilution.

8 *The Effect of Varying Amounts of Complement*.—The results of an experiment performed to determine the variations which occur in the fixation of the complement components when increasing amounts of complement are added to a standard amount of antigen antibody aggregate, show that increasing amounts of complement resulted in a slight increase in the fixation of C′4, a marked decrease in the fixation of C′1, and little or no variation in the fixation of C′3

9 *The Effect of Varying Amounts of Antigen and Antibody*.—The results of

an experiment dealing with the effects of varying amounts of antigen and antibody on complement fixation, show the following: (*a*) in extreme excess of antigen or antibody the fixation of complement or its individual components was markedly inhibited, (*b*) when very dilute solutions of antigen and antibody were used C′4 did not fix completely, (*c*) a slight excess of antibody resulted in maximum fixation of the combining components of complement. Therefore, in the experiments reported here, antibody was employed in a slight excess.

TABLE IV

The Effect of Dilution on the Fixation of Complement

1:8 pneumococcic antiserum	Guinea pig serum	1:40,000 SIII	0.9 per cent sodium chloride solution	Relative quantity and appearance of the precipitate	Hemolysis produced by supernatant after fixation	Hemolysis produced after addition of specifically inactivated complements				
						Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum heated at 56 C for 30 mins.	CO_2-soluble fraction ('end-piece')	CO_2-insoluble fraction ('mid piece')
cc	*cc*	*cc*	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.2	1	0.2	0	++ gran	0	0	60	0	50	70
0.2	1	0.2	1	++ gran	0	0	50	0	30	50
0.2	1	0.2	2	+ gran	0	0	45	0	20	30
0.2	1	0.2	3	+ gran	0	0	45	0	20	30
0.2	1	0.2	4	±	0	0	50	0	20	Tr
0.2	1	0.2	5	0	0	20	60	0	20	Tr
Specifically inactivated complements					Hemolysis					
					per cent					
Serum treated with hydrazine					0	—	60	70	95	50
Serum treated with insoluble carbohydrate					0	60	—	60	35	90
Serum heated at 56°C for 30 mins					0	70	60	—	0	0
"End piece"					0	90	35	0	—	95
"Mid-piece"					0	50	90	0	95	—

The Fixation of Normal Guinea Pig Complement and Specifically Inactivated Guinea Pig Complement.—In order to determine whether the removal of one or more components of complement interferes with the fixation of the remaining components, an experiment such as the one summarized in Table V was carried out. In this experiment normal complement and specifically inactivated complements were added to the specific aggregates of Type III pneumococcus specific substance. The table reveals the following points: (1) Almost all of C′4, 25 per cent of C′3, 75 per cent of C′1, and nearly all of C′2 were removed or inactivated during fixation of normal complement. This is at variance with

the assumption of most workers that the mid piece is the single combining component, as all of the components were fixed in varying amounts under the conditions of the experiment (2) Inactivation of C′4 by hydrazine or the

TABLE V

The Fixation of Normal and Specifically Inactivated Guinea Pig Complements

Pneumococcic antiserum 1:8	1:40,000 SIII	1 cc. of various serums or serum fractions	Hemolysis after fixation	Hemolysis produced after addition of specifically inactivated complements: Serum treated with hydrazine	Serum treated with insoluble carbohydrate	Serum heated at 56°C. for 30 mins.	Serum treated with hydrazine and insoluble carbohydrate	"End piece"	"Mid piece"
cc	*cc.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0 2	0 2	Guinea pig serum	0	5	65	10	10	20	Tr
0 2	0 2	Serum treated with hydrazine	0	0	80	0	0	Tr	0
0 2	0 2	Serum treated with insoluble carbohydrate	0	10	Tr	0	0	0	0
0 2	0 2	Serum treated with hydrazine and insoluble carbohydrate	0	0	10	0	0	0	0
0 2	0 2	Serum heated at 56 C. for 30 mins.	0	95	90	0	75	0	0
0 2	0 2	End piece	0	95	40	0	40	0	45
0 2	0 2	Mid piece	0	0	40	0	0	0	0

Specifically inactivated complements	Hemolysis						
	per cent						
Serum treated with hydrazine.	0	0	65	90	0	95	0
Serum treated with insoluble carbohydrate	0	65	0	90	10	45	60
Serum treated with insoluble carbohydrate and hydrazine	0	0	10	60	0	25	0
Serum heated at 56 C 30 mins.	0	90	90	0	60	0	0
End piece	0	90	45	0	25	0	90
"Mid piece"	0	0	60	0	0	90	0

removal of C′3 by the insoluble carbohydrate from yeast did not markedly influence the fixation of the other components. (3) The inactivation of both C′1 and C′2 by heat inhibited the fixation of C′4 and C′3 (4) When end piece, which contained C′2, C′4, and a small amount of C′3 was added to the specific aggregates, no fixation of C′3 and C′4, and partial inactivation of C′2 resulted Although, C′1 is fixed in the absence of C′4, functionally in so far

as hemolysis is concerned, it is inactive In studies on the mechanism of immune hemolysis (26), it will be shown that unless C'4 is either fixed previously to or simultaneously with C'1, no hemolytic activity occurs (5) All of C'1 but only a small amount of C'3 were removed in the fixation of mid-piece which contained all of C'1 and the major portion of C'3

The results of many experiments similar to the one above show that the direct and immediate inactivation of complement which occurs in fixation is due to the fixation of almost all of C'4 and C'2 and of a part of C'1 C'3 does not appear to play a major part in such fixation reactions However, it appears that the inactivation of the thermostable components (C'4 and C'3) does not interfere with the fixation of the thermolabile components (C'1 and C'2) Further, in the absence of mid-piece, C'2 and C'4 are not adsorbed or inactivated by fixation, while in the absence of C'2 and C'4, C'1 is still adsorbed by specific aggregates The full significance of these results will be elaborated in a subsequent paper dealing with immune hemolysis since they are best interpreted when a visible reaction, such as hemolysis, occurs

The Fixation of the Components by Complement by Various Antigen-Antibody Systems—In order to determine whether the nature of the antigen influences the fixability of the components of complement, a series of experiments was conducted in which antigens of different characteristics and molecular sizes were used

The components of complement were fixed quite similarly by human serum-antihuman rabbit serum, tobacco mosaic virus-antitobacco mosaic virus rabbit serum, and by hemocyanin-antihemocyanin rabbit serum aggregates The fixation of the components by these systems was qualitatively similar to fixation by SIII-antipneumococcus Type III rabbit serum (Table V)

Since sensitized red blood cells of different species are commonly employed in the assay of complementary activity, an experiment was performed in which complement was allowed to fix to the specific aggregates of sheep, dog, human, and guinea pig erythrocytes at 1° C The results obtained were similar to those described for other aggregates, except for a few minor deviations Whereas the fixation of C'4–C'2 in these cases was almost identical with their fixation by other systems, C'3 appeared not to be adsorbed at all Marked variations occurred in the amounts of C'1 which combined with the different red blood cell aggregates, as expressed in the following order guinea pig > sheep > human > dog

DISCUSSION AND SUMMARY

From the experiments reported here it is evident that the amount of each complement component which combines with specific immune aggregates depends upon a number of factors, including the age of the complement, the concentrations of antigen, antibody, and complement, hydrogen ion and electrolyte concentrations, and time and temperature of incubation for fixation

The experiments also reveal the following

1. C′4 must be considered a combining component of complement because it is invariably inactivated or adsorbed by specific aggregates.

2. C′3, although necessary for the final action of complement, *e.g.*, hemolysis or bactericidal action, is fixed only partially or not at all by antigen antibody compounds.

3. C′2 is fixed together with C′4, as is evident from those experiments in which reactivations were conducted with a CO_2-insoluble fraction which contained no C′4 activity.

4. Mid piece, which contains C′1, generally stated to be the single combining component of complement, is adsorbed by immune aggregates in varying amounts depending on the experimental conditions employed.

5. No inactivation or adsorption of C′4 and of C′3 occurs upon the addition to specific aggregates of serum which has been heated at 56°C. for 30 to 50 minutes. This indicates that certain thermolabile constituents of serum are necessary for the fixation of C′4. Inactivation of C′4 by hydrazine or the removal of C′3 by the insoluble carbohydrate from yeast does not markedly influence the fixation of the other components.

6. The assumption that the mid piece is the single combining component of complement is further questioned for the following reasons.

First, C′4 in fresh, untreated serum combines with specific aggregates, but C′4 in heat inactivated serum does not. It is, therefore, apparent that a part of the combining complement must be attributed to C′4 and its carrier C′2. Second, it will be shown in a subsequent paper that C′1, heated at 56°C. for 45 minutes, combines with specific immune complexes, and in doing so may in fact inhibit the further combination of the components of untreated complement. This effect, first noted by Ehrlich and Sachs (27), is termed a "complementoid" action. Thirdly, it will also be shown on the basis of quantitative nitrogen data (28), that the combining nitrogen, in those instances in which large amounts of serum are added to a constant amount of aggregate, should for the most part be attributed to C′4 and its carrier C′2.

7. The nature and the molecular size of the antigen does not influence the qualitative picture of fixation of the complement components.

8. While it is tempting to speculate on the mechanism of complement fixation, it probably is advisable that such speculation be postponed until experiments similar to those reported here are performed with purified components. However, certain differences between the fixation of the complement components to specific aggregates and the adsorption of these components to non-specific agents, are apparent. For example, in specific immune fixation C′4, C′2, and varying amounts of C′1 are fixed, while in non-specific adsorption both to inorganic adsorbents (29) and to untreated bacteria (30, 31), no fixation of C′4–C′2 occurs while all of the other components of complement are adsorbed. Furthermore, C′3 is bound only partially or not at all to specific

aggregates, while it is completely adsorbed or inactivated by non-specific adsorbents It appears, therefore, that the highly reactive component, C′4, is directly and immediately involved in specific immune fixation

9 A very small amount of specific immune aggregate combines with a large amount of complement, whereas a large amount of non-specific agent adsorbs only a small amount of complement This difference is undoubtedly due to the marked chemical affinity of C′4–C′2 and C′1 for the surfaces presented by immune aggregates

10 It can be now stated that when complement exerts its activity in hemolytic, bacteriolytic, or bactericidal reactions, C′4–C′2 and varying amounts of C′1 must first combine (fix) with the antigen-antibody compound in question, and that any secondary manifestation is dependent both on the adjunctive action of the unbound C′3 (26) and on the nature of the substrate employed

The chemical and immunological implications of these results will be further elaborated in subsequent papers

We wish to express our gratitude to Dr Michael Heidelberger for his critical review of this paper

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STUDIES ON MENINGOCOCCAL INFECTION

XII Immunochemical Studies on Meningococcus Type II

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Rake and Scherp (1, 2) have isolated from broth autolysates of meningococcus group I III three principal fractions, one of protein nature and two of carbohydrate nature They have demonstrated that the former carries group-specific properties while of the latter, one has type-specific and the other group-specific properties Little was known about the immunochemistry of Type II meningococcus when the present work was initiated Rake and Scherp (1, 2) had isolated from this type a group-specific protein component and demonstrated that type-specific properties were connected with a certain fraction, which gave both carbohydrate and protein reactions

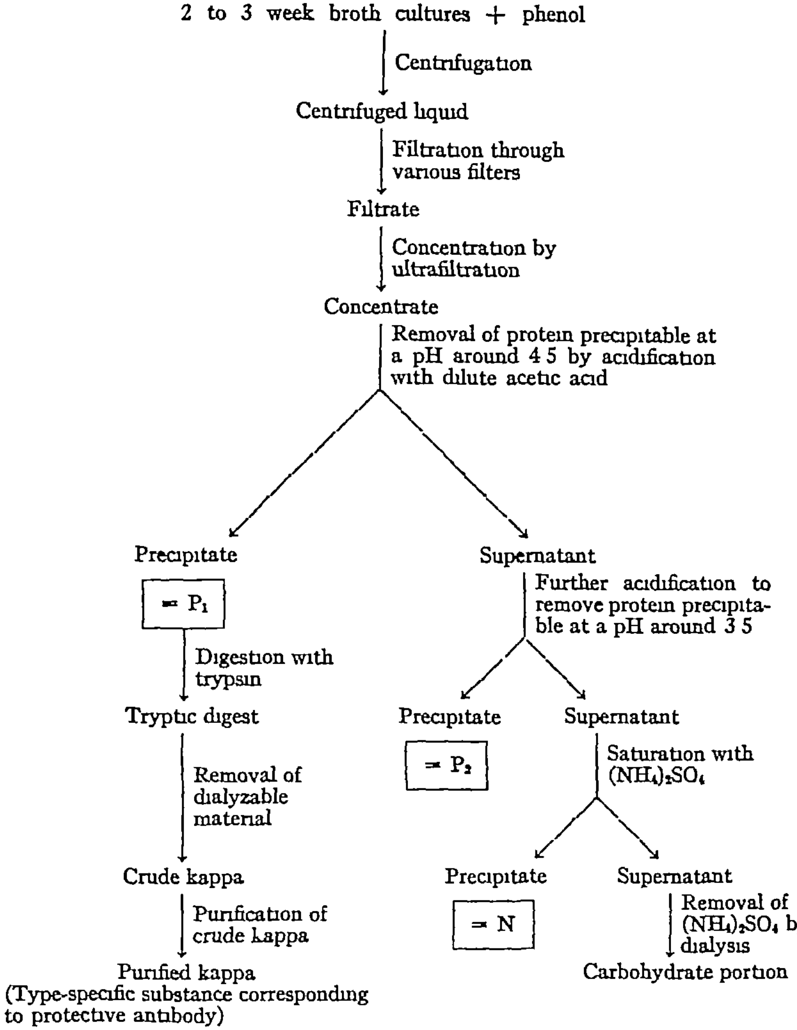
The present work was undertaken to further our knowledge of Type II meningococcus, it was carried out for the most part on a virulent Type II strain (Herrington), and comprised (*a*) studies on autolysates from cultures grown in hormone broth and (*b*) studies on young cultures grown on blood agar

Studies of Broth Autolysates

Broth autolysates were obtained as described previously (1, 2) After phenol to 0.5 per cent had been added they were centrifuged at 30,000 to 35 000 R.P.M. in a Sharples supercentrifuge, and the supernates filtered through various filters to clarify and to remove bacterial debris. Final filtration was always carried out through Chamberland L5 filters. The filtrates were concentrated by ultrafiltration through Bronfenbrenner alundum candles (3) coated with 7 per cent collodion in glacial acetic acid. The concentrates were "washed' on the membrane with distilled water until the filtrates were Molisch negative The filtrates were discarded, as they were found to be serologically inactive 5 filtrates of the 7 different preparations having been tested.

The dark brown colored concentrates may be fractionated as shown in the flow sheet. The P_1 precipitates were further purified by repeated solution in slightly alkalized water, followed by precipitation with dilute acetic acid, until the supernates had become Molisch negative. These steps were carried out in the cold The water washed precipitates were either dried from frozen state

Flow Sheet



on a cryochem apparatus (4) or further washed with acetone, filtered, and dried *in vacuo*

Chemical properties of P_1 fractions obtained from 7 different preparations are summarized in Table I These substances are gray or brown, insoluble in distilled water or saline, but soluble on addition of minimum amounts of dilute NaOH to brown solutions, which flocculate with dilute acetic acid or Tanret's reagent and give positive biuret, Millon, and Sakaguchi reactions

In one instance a similar substance was obtained by acidifying the super-

centrifugate directly This latter method was not used more frequently, however, as ultrafiltration removed broth constituents which otherwise might precipitate together with P_1

As exemplified in the flow sheet, further portions of protein nature (Tanret and biuret positive) can be separated from the supernates of P_1 Type specificity was found to be present not only in P_1, but also in both P_2 and N Data concerning P_2 and N are included in Tables II and V These data, which will be clarified in connection with the experiments described for component P_1, show that P_1 was considerably richer in type-specific substance than P_2 and somewhat richer than N (Table V), N being antigenically less homogeneous than P_1 (Table II)

The deproteinized mother liquors contain portions of carbohydrate nature. Only very weak type-specific serological reactions were given by these polysaccharides when present in high concentrations. Carbohydrate fractions were, however, reactive with polyvalent antimeningococcus horse sera, known to contain antibodies for the group-specific C fraction, and they also showed some reactivity with polyvalent Type 4-8 antipneumococcus horse sera. Such fractions contained about 35 to 37 per cent hexosamine. As peptone was a constituent of the medium it seems reasonable to assume that such portions contain chiefly blood group A substance (from peptone (6)) together with meningococcus C substance and mere traces of type-specific material

TABLE I

Extreme and Mean Values of Chemical Properties of P_1 Fractions Isolated from Various Preparations of the Herrington Strain of Type II Meningococci

	Yield per liter of broth	pH of maximum precipitability	$[\alpha]_D$	N	P	Carbohydrate	Ash as Ca
	mg.			*per cent*	*per cent*	*per cent*	*per cent*
Highest value	234 2	3 9	−58	14 6	0 9	3 2	0 24
Lowest value	93 0	4 7	−38	13 1	Trace	1 8	0 15
Mean	162	4 4	−46	13 7	0 4	2 8	0 2

* The method of Heidelberger and Kendall (5) was applied and the colors, read in a photoelectric colorimeter evaluated from equations calculated from galactose standards.

For these reasons P_2, N, and carbohydrate components were studied less thoroughly

Serological Studies

For serological studies the ring test technique was applied throughout, the readings recorded in the tables are those taken after 2 hours at 37°

The following antimeningococcus Type II sera were used (1) A pool from several

bleedings of two rabbits designated 13-15, these animals had been immunized with whole cultures of Type II meningococcus and the mouse protective value of this pool was 67 units (2) Another pool, also from several bleedings of two rabbits and designated 3185-3192, exhibited 180 mouse protective units For certain tests the latter serum was exhaustively absorbed with young Type I organisms grown on blood agar, and was then designated "3185-3192 absorbed." (3) Three more sera (M18, M22, and M37) were prepared by immunizing rabbits with 6 hour cultures of Type II meningococci The mouse protective values of these three sera were 16, 47, and 27 units respectively (4) Similarly, anti-P_1 sera were prepared by immunizing rabbits with 1 1000 saline dilutions of a P_1 fraction, starting usually with 0 1 ml. and increasing the dose gradually to 1 0 ml. P_1 proved to be quite toxic, as evidenced by temperature reactions and by several fatalities Anti-P_1 sera gave strong precipitin reactions with meningococcal proteins (*cf* Tables V and X), but were of only low mouse protective value, the highest potency observed was 9 units They reacted also with acid-precipitable protein derived from Type I (Table V) but exhibited, on the other hand, a certain degree of type specificity in the agglutination reaction

In serological respects, P_1 exhibited two important properties (1) It reacted strongly with both antiprotein serum and (absorbed) antimeningococcus Type II serum 3185-3192, as will be seen from data recorded in Table V During the course of experiments attempting to separate the type-specific fraction from the protein it was found that the reactivity with antiprotein serum was lost after digestion with proteolytic enzymes while the reactivity with serum 3185-3192 was retained fully or even increased These experiments will be described in detail later (*cf* page 443, and Table V) (2) It removed from protective serum (13-15) all protective antibody Thus samples of serum 13-15 were absorbed with various P_1 fractions at the antigen excess end of the equivalence zones, the absorptions were carried out by keeping the mixtures in the cold and shaking occasionally for 4 days All the P_1 fractions from Type II removed all protective antibody, a protein component derived from Type I and comparable to P_1 in so far as it was acid-precipitable and prepared in an analogous manner, failed to do so The data of these experiments are recorded in Table II

As mentioned above, we have reason to believe that P_1 is richer in type-specific substance than either P_2 or N Accordingly, attempts were made to separate the type-specific principle from P_1 protein However, contrary to the experiences with Type I meningococcus (1, 2, 7), we could not separate the type-specific substance from protein by repeated acid precipitation The following methods also failed to separate type specificity from "accompanying" protein (*a*) treatment with $CHCl_3$ and butyl alcohol, according to Sevag (8), at neutrality and at slight alkalinity, (*b*) heating P_1 in diluted Na_2CO_3 solution at 50 to 55°C , followed by neutralization and repeated treatment with $CHCl_3$ and butyl alcohol, according to Sevag, Lackman, and Smolens (9), (*c*) subfractionation with the aid of $(NH_4)_2SO_4$, (*d*) electrophoresis in a 7 cell electrophoresis apparatus (10)

a Saline solutions of a P_1 preparation (BII^9P_1) were treated with $CHCl_3$ and butyl alcohol. The aqueous phases were tested serologically. Protein removal was paralleled by loss of all serological reactivity.

TABLE II

Precipitin Reaction of Various Fractions with Serum 13-15 (67 Mouse Protective Units) and Mouse Protective Units Remaining in the Serum after Absorption

		Reactivity of absorbed serum with fraction used for absorption and with unabsorbed serum									
		BIIP$_1$‡	13-15	BIIP‡	13-15	BIIP$_1$‡	13-15	BIIN§	13-15	BPI‖	13-15
Serum absorbed with equal volume of saline dilution 1	100	±	++±	−	++±	−	++	++	(∓)		
	250	+	+±	(±)	++	(∓)	±	++	(∓)	−	+±
	500	+±	+	+	+±	(∓)	(±)	++±	(∓)	(∓)	±
	750	++	(±)	+±	+(±)	±	(∓)			(∓)	(±)
	1000	++±	(∓)	+±	+	+±	−			(±)	(∓)
Serum absorbed with equal volume of dilution 1		200		300		200		100		600	
Mouse protective units in absorbed serum		<5**		<10**		<4**		<5**		56	

* In this and the following tables the following denotations are used

(∓) questionable reactions.
(±) very weak but still definite reactions.
± weak but definite reactions.

+, +±, ++, ++±, +++, +++±, ++++ = increasing amounts of precipitate.

‡ Laboratory designation of P_1 fraction used.
§ Laboratory designation of N fraction.
‖ Laboratory designation of acid-precipitable protein component prepared from Type I, the dilutions used in this instance were 1 300 600 900 and 1200 respectively.
** Least value tested.

b About 100 mg of a P_1 preparation ($BII^{10}P_1$) were treated under the conditions described by Sevag, Lackman, and Smolens (9). Aliquots were withdrawn before and in the course of $CHCl_3$ treatment and tested serologically, as recorded in Table III. From the combined $CHCl_3$ layers a substance was isolated by addition of redistilled acetone. This substance was of protein nature, as evidenced by its preparation and by its being biuret positive. It was tested before and after tryptic treatment with antiprotein serum and with

TABLE III

P_1 Treated According to Sevag, Lackman, and Smolens (9) Parallelism of Protein Removal with Decrease of Serological Reactivity

Conditions	No of treat ments	Vs antiprotein serum M2-1.5.39			Vs 3185-3192 absorbed		
		1T	5T	10T	1T	5T	10T
Untreated		++++			+++		
Heated at 50 to 55°C in diluted Na_2CO_3 solution	0	+++±			+++		
	2	+++			+		
	4	++			(±)		
	6	+±			(∓)		
	8	+			−		
	10	+			−		
	12	±			−		
	14	±			±		
Tryptic digested after 14 treat ments*	14	(±)			±		
Substance isolated from $CHCl_3$ phase							
Before trypsin		+++±	+++	++	+	±	−
After trypsin		±	−	−	+++	++	+±

* Dilution 1 2.5T

TABLE IV

Cell Contents after 21 Hours Electrophoresis of P_1 and Serological Tests on Them

Cell No	1 (Cathodic)	2	3	4		5	6		7	
Appearance	Water clear color less	Water clear color less	Water clear color less	Water-clear supernate over flocculent precipitate		Water clear colorless	Slightly turbid		Slight flocculent precipitate	
				Supernate	Redissolved precipitate		Supernate	Redissolved precipitate	Supernate	Redissolved precipitate
Biuret	−	−	−	±	+++	−	−	−	−	+
pH	9 6	8 0	6 8	6 2		5 9	5 5		3 6	
Vs antiprotein serum M2–12 14 38	−	−	−	−	+++± (±)*	−	−	−	−	++ −*
Vs serum 3185-3192 absorbed	−	−	−	−	+± +++*	−	−	−	−	(±) +*

* After digestion with trypsin.

absorbed 3185 3192 serum, as recorded in Table III This experiment shows again that the active principle is firmly bound to protein, as it disappeared gradually from the aqueous phase and could be found in the protein portion isolated from the $CHCl_3$ layer

c 820 mg of a P_1 preparation (BII^9P_1) were fractionated with $(NH_4)_2SO_4$. Three fractions of protein nature were obtained, precipitable at 23 per cent, 45 per cent, and 100 per cent saturation in yields of 317, 143, and 130 mg respectively These fractions gave positive biuret and Sakaguchi reactions. Only the portion precipitable at 23 per cent saturation gave a distinct Millon test. All three were reactive with both antiprotein serum and with absorbed 3185-3192 serum, and all three removed nearly all protective antibody from serum 13-15 (67 units) From the final $(NH_4)_2SO_4$ supernates a small, serologically inactive, biuret negative, and strongly Molisch positive fraction (64 mg) was obtained This experiment also shows that the active principle is connected with protein

d A dialyzed solution of 40 mg of a P_1 preparation (BII^9P_1) in 20 ml distilled water was subjected to electrophoresis in a 7 cell apparatus (10) at 2,000 volts The substance was placed in the middle cell, the other cells being filled with distilled water After electrophoresis for 21 hours, the picture, including biuret and serological tests, was as recorded in Table IV This experiment shows that, on electrophoresis, partial migration and separation take place, but that serological reactivity again is only connected with those portions which also are biuret positive, *i.e.*, only with components of protein nature. Two more electrophoresis experiments on another P_1 fraction ($BII^{10}P_1$) and on a P_2 fraction (see flow sheet) led to the same conclusions.

Action of Enzymes on P_1—Kappa Substance

Under the influence of proteolytic enzymes P_1 loses its reactivity with anti-P_1 serum, while its reactivity with (absorbed) antimeningococcus Type II serum is fully retained or even intensified, as exemplified by the following experiment

Two 1 0 ml. samples of an aqueous solution of a P_1 fraction (BII^7P_1) containing 159 mg. per 15 ml. were pipetted into Wassermann tubes To each sample 1 0 ml. M/5 phosphate buffer pH 7 0 and 2 drops of toluene were added. To one of the tubes 0.25 ml of a trypsin solution (250 mg /10 ml.) which had been roughly purified by removing acid precipitable material, was added. The tubes were capped and placed into a water bath at 37° for 4 days. Saline dilutions corresponding to 1 1000 1 5000, and 1 10 000 of the substance originally used were made up and tested against anti P_1 serum and against absorbed 3185-3192 serum.

All of the P_1 fractions were tested according to this general scheme. The mean results are recorded in Table V Trypsin controls which had been incu-

bated in absence of P_1, reacted neither with anti-P_1 serum nor with absorbed 3185-3192 serum

The results of numerous other enzymatic experiments (Table V) may be summarized as follows (*a*) Serial addition of trypsin up to 2 ml trypsin (*i e* 50 mg) per 10 mg P_1 and incubation up to 10 days did not alter the results in any way (*b*) Identical results were obtained when tryptic digestion was

TABLE V

Reactivity of Meningococcus-Protein Fractions with Antiprotein Serum and with Absorbed Antimeningococcus Type II Serum

Laboratory designation of fraction	Enzyme	Vs antiprotein serum						Vs anti Type II serum, absorbed					
		Before enzyme			After enzyme			Before enzyme			After enzyme		
		1T	5T	10T	1T	5T	10T	1T	5T	10T	1T	5T	10T
P_1 (Mean of 7 preparations)*	Commercial trypsin‡	++++±	+++±	++(±)	+(±)	±	−	++±	+(±)	+	++++±	++(±)	++±
BII^{1}P_1	Activated papain at neutral reaction§				++	++±	+				++++±		++±
BII^{11}P_1	Crystalline pepsin at pH 3 0‖				±	−	−				++++±	+++±	++
BI^{3}P_1**	Commercial trypsin	+++±		++±	(±)		−	−		−	−		−
		500	5T	10T	500	5T	10T	500	5T	10T	500	5T	10T
BII^{8}P_2	Commercial trypsin	+++	++		+	±		++	−		+++±	−	
BII^{8}N	Commercial trypsin	+++±	±	±	++	±	±	++++±	+	±	++++±	+	±

* Similar results on P_1 from a culture grown on a semisynthetic medium, containing casein-hydrolysate and yeast extract as principal ingredients

‡ Similar results when digested at pH 7 6, 8 5, or in N/4 Na_2CO_3 Similar results with crystalline trypsin, crystalline chymotrypsin, or mixtures of both at neutral reaction

§ Similar results on three other fractions digested at pH 5.2

‖ Similar results when digested in N/10 HCl

** Acid precipitable protein component prepared from Type I

carried out in saline solution or at pH values of 7 6 or 8 5 or in N/4 Na_2CO_3 solution (*c*) Activated papain at neutral reaction and at pH 5 2 caused essentially the same effect, although the destruction of anti-P_1 serum reactivity was less marked (*d*) Crystalline pepsin acted at pH 3 0 and in N/10 HCl solutions essentially the same way as trypsin at the conditions described

It appeared evident that the type-specific principle could not be removed from protein by mild, simple, and but slightly destructive means As, however, this principle was found in enzymatic digests of P_1, we attempted to iso-

late this substance, termed kappa substance, from tryptic digests. By purifying tryptic digests with charcoal, followed by dialysis,[1] fairly colorless kappa was obtained (*cf* Table VI), as, however, by repeated charcoal treatment eventually all serological reactivity was lost, the following method of purification was chosen

P_1 from 4 different preparations was pooled and digested with trypsin in presence of phosphate buffer pH 7 5 The digest was freed from phosphate by dialysis and brought to dryness on a cryochem apparatus. 1 8 gm. of crude kappa substance were obtained from 9 4 gm. P_1 On the basis of the precipitin

TABLE VI

Chemical and Serological Properties of Crude Kappa, Purified Kappa, and of Component Separated from Crude Kappa by $(NH_4)_2SO_4$ Fractionation

	$[\alpha]_D$	N	P	Carbohydrate	Serological reactivity with: Anti-protein serum			3185-3192, absorbed			
					1T	10T	100T	1T	10T	100T	1M
		per cent	*per cent*	*per cent*							
Crude kappa		9 0	1 5	8 9	+	(±)	−	++++	+++	+	
Purified kappa		9 6	1 4	12 3	(∓)	−	−		+++±	+±	(∓)
		9 6		12 9	(±)	−			+++±	+±	−
Main component of the $(NH_4)_2SO_4$ supernate	+7 5	8 7	1 7	7 8	−	−	−	+±	(∓)	−	
	+13 5	8 1		7 0	(∓)	−	−	+	−	−	
	+14 1	6 8			−	−		+	−		
Kappa obtained by charcoal treatment	+2	5 2			+±	−		+++±	++±	±	

test there was approximately a tenfold increase in activity from P_1 to crude kappa.

Such crude kappa substance was further purified by removal of a small portion, insoluble in chilled 0.2 M acetate buffer pH 4 0, followed by $(NH_4)_2SO_4$ fractionation[2] At 62.5 per cent $(NH_4)_2SO_4$ concentration, the bulk of active material flocculated out. Material isolated from the supernate showed very

[1] 250 mg. trypsin autodigested in presence of phosphate buffer and dialyzed against distilled water yielded after drying from frozen state a residue too small to be collected. This small residue dissolved only partially in 0 2 M acetate buffer at pH 4 0, the clear centrifuged supernate rendering only a faint opalescence on saturation to 62.5 per cent with $(NH_4)_2SO_4$ Trypsin may therefore be excluded as tangible impurity in any of our kappa preparations.

[2] $(NH_4)_2SO_4$ being removed subsequently by dialysis.

little serological reactivity The portion precipitable at 62 5 per cent $(NH_4)_2SO_4$ was repeatedly treated with $CHCl_3$ and butyl alcohol, according to Sevag (8), when only a small amount of material was removed into the $CHCl_3$ phase The aqueous phase was brought to dryness on a cryochem apparatus The yield was 172 mg purified kappa from 727 mg crude kappa In addition, 169 mg "almost inactive" material from the $(NH_4)_2SO_4$ supernates, and (active) minor fractions, aggregating to 93 mg, were isolated, the $CHCl_3$ phase rendered 10 mg of (active) material In another experiment, 177 mg purified kappa were obtained from 545 mg crude kappa On the basis of the precipitin test, there was only little increase in serological activity from crude kappa to purified kappa

Purified kappa consists of a grayish brown powder, readily soluble in distilled water or saline to give a somewhat opalescent solution, which clears up on addition of minimal amounts of diluted NaOH It is Tanret, Sakaguchi, and ninhydrin-positive but biuret-negative Chemical and serological properties are recorded in Table VI

When tested in the precipitin reaction with three different anti-Type I sera, kappa was found non-reactive with one of these sera, while weak reactions (++ and + respectively in 1 1T, and + and ± in 1 10T) were observed in the other two sera[3] It is not known whether these weak reactions are due to small amounts of an impurity or to the "existence of antigenic determinants of diverse specificity" in the kappa molecule, in the sense of Hooker and Boyd's observation on systems of crystalline duck and egg albumin and their antibodies (11)

Kappa removes practically all protective antibody from Type II protective antisera, as shown in Table VII Quantitative nitrogen determinations performed according to the method of Heidelberger and Kendall (12–14) on the precipitates of these experiments, indicate a correlation between total precipitated nitrogen and mouse protective value

Electrophoresis experiments carried out both in the Tiselius apparatus,[4,5] and in a 7 cell apparatus (10) did not free kappa from colored matter Part of the color at least seems, nevertheless, not to be an integral part of the molecule, as supernates of serum M22 pooled-1, absorbed with kappa at the equivalence point, were distinctly brown colored, but did not react with unabsorbed serum Possibly electrophoresis in the Tiselius apparatus at other pH values might have led to separation of colored matter from kappa Lack of material

[3] Weak heterologous reactions of the same order of magnitude had also been observed with the mother substance of kappa, *i e*, P_1

[4] We wish to express our thanks to Dr D Moore, of the Presbyterian Hospital in New York, and Dr B Chow, of this Institute, for carrying out these tests

[5] Kappa being dissolved in 0 02 M phosphate buffer pH 7 45 and pH 7 47 respectively

has prevented us from such studies. As the color of kappa interfered in the Tiselius apparatus with observation and photographic recording the question about the electrophoretic homogeneity of kappa remains still unanswered.

In the experiment conducted by Dr Chow, small quantities of a fast moving colored portion (*a*) and a slow moving, slightly colored portion (*b*) were collected and isolated.[8] Both were equally reactive with absorbed serum 3185-3192 By further absorption of this serum with (*a*) all reactivity with (*b*) and with kappa had disappeared. When kappa was subjected to electrophoresis

TABLE VII

Equivalence Zone Determination of Various Anti-Type II Sera with Kappa and Mouse Protective Units Remaining after Absorption at Antigen Excess End of Equivalent Zone

Serum absorbed		M18-pooled-1 (16MPU)		M22-pooled-1 (47MPU)		M37-11.22.40 (27MPU)	
		Reactivity of absorbed serum with kappa or with unabsorbed serum					
		Kappa	M18-pooled-1	Kappa	M22 pooled-1	Kappa	M37-11.22.40
Serum absorbed with equal volume of saline dilution 1	320			(∓)	++		
	500					−	+++
	640			±	−		
	1T			+++±	−	−	+±
	1.5T	(±)	+±			−	±
	2.0T	(±)	±			(±)	−
	2.5T	±	−			(±)	−
2.0 ml. serum absorbed with 1.0 ml. saline dilution of kappa 1		750		239		750	
Mouse protective units remaining in absorbed serum		4		4		3	
Mg total precipitated N_2 per ml. serum		0 175		0 533		0 278	

from the cathodic end-cell of the 7 cell apparatus (10) migration took place through the whole system into the anodic end-cell. After 21 hours the main portions of kappa had accumulated in the cathodic and anodic end-cells, as indicated by the color and as confirmed by weight determinations on the isolated cell contents. No serological cross reactions occurred, when samples of absorbed serum 3185-3192 were further absorbed with these main portions of kappa which had been subjected to electrophoresis, and were cross tested in

[8] We must leave the question open whether kappa separated under the experimental conditions into 2 (or more) portions, or whether the appearance of a colored, fast moving, and a but slightly colored slow moving portion was due to the superimposed effect of colored matter

the precipitin reaction. The fact that electrophoresis effected no separation into serologically different fractions indicates a certain degree of purity for kappa Neither the experiments in the Tiselius apparatus nor those in the 7 cell apparatus gave any indication of separation of serologically active carbohydrate from kappa Carbohydrate appears to be rather in chemical linkage to the rest of the molecule This point is supported by the fact that serum-kappa precipitates are always of the finely granular type, which usually is interpreted as a protein-antiprotein reaction, compact discs, which are considered as indicative of carbohydrate-anticarbohydrate, were never observed Considering the various data, kappa itself appears as a protein split-product, carrying some colored matter as an impurity which can be separated only with difficulty The protein nature of kappa is manifested by positive ninhydrin and Sakaguchi tests and is indicated by the electrophoretic mobility in the Tiselius apparatus which is in the order of $10^{-5} \frac{\text{cm /sec}}{volt/cm}$ Besides amino acids, there appears to be carbohydrate present as part of the molecule The bulk of kappa, therefore, can be considered to be a carbohydrate-containing polypeptide

Studies on Young Cells

As we had isolated this kappa substance from broth autolysates, we were interested in ascertaining whether we could identify the protein from broth autolysates with protein from young meningococci Type II cultures Five to 6 hour blood agar cultures were fractionated according to the method developed by Heidelberger and Kendall (15) on a scarlatinal strain of hemolytic streptococcus and later applied by Heidelberger and Menzel (16) in their studies on tubercle bacilli Save for slight, unessential modifications in the procedure, methods and designations are the same in the present work The properties of the main fractions (D and K) obtained in two different preparations are compiled in Table VIII

These quantitatively most abundant fractions were used to absorb protective serum 13-15 in the same manner as described above This serum contained originally 67 units The results are recorded in Table IX

Furthermore, the various fractions were subjected to tryptic digestion and serological tests performed as described above The results upon D and K are recorded in Table X

The absorption experiments show that specificity corresponding to protective antibody is predominantly connected with fraction K, although this specificity is encountered to a considerably lesser degree also in fraction D, the experiments on tryptic digested fractions show clearly a basic difference between D and K. The serological, as well as the chemical properties, of the several intermediate fractions lie broadly between those of D and K It

TABLE VIII

Chemical and Immunological Properties of Protein Fractions D and K of Type II Meningococci

Preparation	Fraction	Yield	pH of extraction	pH at which precipitated	pH of solution used for $[\alpha]_D$ determination	$[\alpha]_D$	N	P	Carbohydrate	Basic ash as Ca
		gm.					*per cent*	*per cent*	*per cent*	*per cent*
BII⁶	D	1 966	7 0	3 7–3 9	6 7	+12 3	15 7	3 1	10 0	0 1
	K	0 731	$\frac{N}{10}$ NaOH	4 5–5 3	6 7	−45 7	13 3	0 5	2 1	0 0
BII⁶ᴬ	D	1 358	6 8	3 6–4 0	7 5	+27 0	16 0	3 7	11 1	0 3
	K	1 256	$\frac{N}{10}$ NaOH	4 6–4 8	6 8	−41 1	13 8	0 8	2 2	0 2

TABLE IX

Equivalence Point Determination of Various Fractions from Whole Cells with Serum 13-15, and Mouse Protective Units Remaining after Absorption at Equivalence Point

		Reactivity of absorbed serum with fraction used for absorption and with unabsorbed serum, respectively							
		BII⁶D	13-15	BII⁶ᴬD	13-15	BII⁶K	13-15	BII⁶ᴬK	13-15
Serum absorbed with equal volume of saline dilution 1	100	+	+++	−	++(±)	−	+++		
	250	+++	+±	++±	++	−	+++	−	+++
	500	+++±	+	+++±	(±)	−	++	−	++
	750	++++	±	+++±	−	+	+	−	+
	1000	++++	±			+	±	(±)	±
	1250	++++	(±)			+	−	±	(±)
	2500	++++	−			++	−	+	−
Serum absorbed with equal volume of saline dilution 1		100		100		500		750	
Mouse protective units in absorbed serum		57		24		<5*		<4*	

* Least value tested.

TABLE X

Reactivity of Various Fractions Derived from Young Mass Cultures before and after Tryptic Digestion

Fraction	Vs. antiprotein serum						Vs anti-Type II serum absorbed					
	Incubated control			After trypsin			Incubated control			After trypsin		
	1T	5T	10T	1T	5T	10T	1T	5T	10T	1T	5T	10T
BII⁶D	++±	+±	+	+	−	−	++	+	(±)	±	−	−
BII⁶ᴬD	+++	++	+	(∓)	−	−	+++	±	−	±	−	−
BII⁶K	+++±	++±	++±	++	+	+	+++±	++±	+±	+++±	++	+±
BII⁶ᴬK	+++±	++±	++	++	+	±	+++±	++±	+±	++++	++±	++

is, therefore, possible that those intermediate fractions are mixtures of "ideal" D and K No indication of the presence of type-specific substance was found in the acid extracts of the meningococci in the precipitin test with absorbed 3185-3192 serum, the final conclusion being that specificity corresponding to protective antibody in young cells is also intimately connected with protein, especially that protein portion (K) which requires the highest alkali concentration to be brought into solution

It seemed conceivable that by immunizing rabbits with K, sera of high protective values could be obtained This was not the case The highest potency ever observed in 3 animals was 8 units Precipitins against meningococcal proteins were, however, obtained in high titer

DISCUSSION

The preceding experiments show that in meningococcus Type II Herrington strain, type specificity corresponding to protective antibody is intimately connected with protein In this respect meningococcus Type II differs strikingly from Type I and also differs from the various strains of pneumococci

Rake and Scherp (1, 2) have isolated from Type I broth autolysates a protein component, which on account of its acid precipitability might be compared with P_1 of the present studies Both components occur uniformly in the step of acid precipitation, although neither is believed to be a chemically pure entity P_1 removes from protective anti-Type II serum all protective antibody, it is furthermore reactive with absorbed 3185-3192 serum before and after tryptic digestion, the acid-precipitable component from Type I, on the other hand, lacks all these qualities (Tables II and V)

It appears that type specificity of Type II, as manifested by correspondence to the protective antibody, is firmly connected with protein Also in young cells type specificity occurs in connection with protein constituents, predominantly with that protein portion which requires the highest alkali concentration to effectuate dissolution

In broth autolysates this type-specific substance appears to be connected with various components of protein nature We have used P_1 as an easily accessible starting material for isolation of this substance, although possibly other protein portions (P_2 or N of the flow sheet) could be used to advantage for this purpose The kappa substance could be split from P_1 by tryptic digestion and further purified by methods described above. Although this substance has not as yet been obtained in absolutely pure state, we feel that the purity arrived at indicates that the bulk of kappa is composed of a carbohydrate-containing polypeptide The chemical linkage of the phosphorus still remains to be ascertained

The term "type-specific substance," is usually connected with the idea of polysaccharides, although a few examples of type specificity in connection with protein or protein-like substances are known Such instances are well estab-

lished in Group A hemolytic streptococci (17–19, 15) and in Type A staphylococcus (20). Type specificity is completely destroyed by proteolytic enzymes in the cases of Lancefield's M substance (18, 19) and Verwey's staphylococcal protein (20). In the instance of meningococcal protein, however, this is not so. Kappa substance indeed behaves as if it constitutes an enzyme-resistant prosthetic group to meningococcal protein. For this reason, we believe that we are dealing with a new kind of type-specific substance, different from the type-specific polysaccharides and also different from the above discussed type-specific proteins.

Polysaccharidic antigens, which also contain some polypeptide as part of their molecule, are known, for instance the specific antigen obtained from *B. dysenteriae* (21). Therefore, kappa would seem to belong in the middle of a sequence of type-specific substances varying from proteins to polysaccharides as follows: (1) proteins, all specificity destroyed by enzymes, (2) proteins, type specificity connected with an enzyme-resistant prosthetic group, comprising polypeptides plus some carbohydrate, (3) polysaccharides containing some polypeptide, (4) polysaccharides, polypeptide-free. Such a scheme would find further confirmation in the fact that results somewhat analogous to ours have been obtained by Henriksen and Heidelberger by the tryptic digestion of protein fractions of the hemolytic streptococcus (22).

Our findings differ from the results of Miller and Boor (23). These investigators are of the opinion that the type-specific substance of Type II meningococcus is of lipocarbohydrate nature, comparable to the substances isolated by Boivin and Mesrobeanu (24) from *B. aertrycke* and other microorganisms. This discrepancy, however, may find an explanation, in the fact that Miller and Boor have worked with strains of meningococcus Type II different from our strain.

Experiments carried out by us on strains other than the Herrington strain, namely the Albany strain 36 and two strains of Miller and Boor's, designated 44 and 45, indicated that only the Albany strain 36 resembled closely the Herrington strain, in that from it an acid-precipitable P_1 could be obtained which chemically and serologically resembled P_1 from the Herrington strain. Autolysate concentrates of strains 44 and 45 were reactive with both antiprotein serum and with absorbed anti-Type II serum, losing the former but retaining the latter reactivity after digestion with trypsin. Acid-precipitable portions could not be obtained from such autolysate concentrates, but on saturation with $(NH_4)_2SO_4$ to 33.3 per cent precipitates resulted, which serologically, although not chemically, resembled the P_1 fractions obtainable from Herrington strain preparations. These facts certainly indicate the presence of kappa substance also in strains 44 and 45.

CONCLUSIONS

From meningococcus Type II broth autolysates, a substance, corresponding to the protective antibody has been isolated. This substance, termed kappa

substance, is firmly connected with protein but can be separated by the action of proteolytic enzymes Methods of preparation and purification are given, together with chemical and serological data In whole cells from young cultures, this substance is also connected with protein, especially with one protein fraction which requires highest alkali concentration to effect solution

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STUDIES ON HYPOALBUMINEMIA PRODUCED BY PROTEIN-DEFICIENT DIETS

I. Hypoalbuminemia As a Quantitative Measure of Tissue Protein Depletion*

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The realization within recent years of the prevalence and seriousness of protein deficiency has emphasized the need for some simple means of recognizing the presence and degree of such a condition. The only proteins capable of direct clinical measurement are those in the blood. Yet there has, so far, been no evidence that a lowered plasma protein concentration reflects accurately anything but a deficiency in the blood alone.

During the course of other experiments, evidence was adduced indicating the existence of a constant relationship between losses in the total circulating plasma albumin and in the body protein as a whole. This, of course, immediately suggested a means of recognizing and evaluating total body protein deficiency Of the plasma proteins it is only the albumin fraction which consistently decreases during an inadequate protein intake (1–3), for this reason consideration may be confined to this fraction alone. The total circulating serum[1] albumin can be readily obtained by multiplying the albumin value by the plasma volume which may be estimated from the body weight or determined directly By subtracting this total circulating serum albumin from the normal, the number of grams of albumin by which the serum is depleted is obtained The depletion in the tissue protein is, of course, readily measured by determining the total nitrogen loss in the urine and feces. Now if the relationship between total serum albumin depletion and total tissue protein depletion is represented by the equation $\frac{\text{Total serum albumin depletion}}{\text{Total protein depletion}} = K$, the value of K is readily calculated. On the other hand if K is known and the serum albumin measured the only unknown would be the tissue protein depletion for which the equation could then be solved.

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[1] Because fibrinogen comes out in the globulin fraction, serum albumin and plasma albumin are practically the same, the terms will be used interchangeably in this paper

TABLE I

Data Showing the Approximate Quantitative Relationship (K) between Dietary Changes in Total Serum Albumin and Tissue Protein

Author	Dietary procedure	Plasma volume	Loss of serum albumin and tissue protein: Total albumin loss (gm.)	Total tissue protein loss (gm. total urinary N × 6.25)	$K\left(\frac{\text{serum albumin loss}}{\text{tissue protein loss}}\right)$
Weech, Goettsch, and Reeves (2)	Low protein—carrot diet 30 days	Assumed 1/20th of body weight	Not recorded	Not recorded	0 04 (approximate)
Holman, Mahoney, and Whipple (4) Dog 22-131	Glucose by gavage for 24 days	Directly determined (dye)	11 9	292*	0 04
Madden *et al* (5) Dog 39-223	Fasted 1 wk.	Directly determined (dye)	3 28	82 6	0 04
Madden *et al* (5) Dog 39-234	Fasted 1 wk.	Directly determined (dye)	5 14	101 25	0 05
Present data	Low protein diet for 21 days	Assumed 1/20th of body weight	5 28	176	0 03
Average					0 04
			Gain in serum albumin and tissue protein: Total albumin gain (gm)	**Total tissue protein gain (gm. N retained × 6.25)**	$K\left(\frac{\text{serum albumin gain}}{\text{tissue protein gain}}\right)$
Elman, Sachar, Horvitz, and Wolf (6) average of 4 dogs	4 gm of casein hydrolysate per kg per day by mouth for 1 wk.‡	Assumed 1/20th of body weight	1 76	71 87	0 02
Average of 5 dogs	4 gm of casein hydrolysate per kg per day for 1 wk. intravenously‡	Assumed 1/20th of body weight	1 4	32 6	0 04

* Loss in last 5 days estimated

‡ Calories supplied with karo syrup

In this paper we shall present evidence indicating that such a constant relationship between the total circulating serum albumin and the tissue proteins does exist under conditions of inadequate protein intake, and that in dogs at least the value for K is in the neighborhood of 0 04 Indeed, there are reasons for believing that the same equation applies to the distribution of nitrogen intake during replenishment of the depletion

Although no previous studies could be found devoted to the topic of this communication data of several workers have been used in calculations which seem to confirm the thesis presented in this paper These calculations are simple. Total serum albumin loss is obtained by multiplying the plasma volume by the change in the concentration of serum albumin nitrogen during the experimental period. The loss in tissue protein was measured by determining the total negative nitrogen balance during the same period. By comparing these two figures of nitrogen loss the proportion of total nitrogen loss was obtained which could be accounted for by the serum albumin change. When the plasma volume was not actually determined, it was assumed to be one twentieth of the body weight.

Weech, Goettsch and Reeves (2) noted that about 4 per cent of the nitrogen lost by dogs on a low protein diet could be accounted for by the decline in total circulating serum albumin Calculations made of the data presented in the papers of Holman Mahoney and Whipple (4) and of Madden Zeldis Hengerer Miller Rowe, Turner, and Whipple (5) showed a decline in the total circulating albumin of fasted dogs which also represented about 4 per cent of the total nitrogen lost. It was interesting to note that the same percentage was reached if one calculated in the same way data obtained while regeneration of serum albumin was produced by feeding protein. During the correction by protein alimentation in experimentally induced hypoproteinemia of dogs, Elman Sachar Horvitz, and Wolf (6) found that only about 4 per cent of the nitrogen which the dogs retained could be accounted for by the increase of serum albumin thus produced. These previous observations are summarized in Table I.

PRESENT OBSERVATIONS

Five dogs were placed on a protein-poor diet containing karo syrup Ringer's solution and a vitamin B complex concentrate for 3 weeks. A constant intake was achieved by feeding the dogs by gavage twice daily a solution prepared as follows 200 gm. of a vitamin B complex concentrate (labco)[2] and 5 000 gm. of karo syrup were made up to 10 000 cc. with 4 times concentrated Ringer's solution Each feeding consisted of enough of this stock solution to provide 25 calories per kilo body weight *i.e.*, 12.5 cc. per kilo diluted 4 times with water and given by gavage. The total daily energy intake was therefore 50 calories per kilo

Plasma albumin determinations and concomitant nitrogen balance studies were carried out while the dogs were on the diet described above The chemical and experimental procedures have been described in a previous paper (6)

[2] Obtained from the Borden Company Bainbridge, New York.

EXPERIMENTAL FINDINGS

The results of the present experiments are tabulated in Table II and are really self-explanatory In brief they show that of the total nitrogen lost by the dogs during 3 weeks on a low protein diet, a relatively small but constant percentage averaging about 3 per cent, represented the fall in serum albumin. The calculations employed were those previously described The value found in these experiments compares fairly well with those derived from the literature as tabulated in Table I.

TABLE II

Quantitative Relation (K) between Total Tissue Protein Loss and Serum Albumin Loss during 3 Weeks of a Protein-free Diet (Plasma Volume Assumed to be 1/20th of Body Weight)

Dog	Loss in body weight	Fall in serum albumin con centration	Loss in total serum albumin	Loss in total tissue protein (gm. total urinary N × 6.25)	$K = \frac{\text{loss total serum albumin}}{\text{loss total tissue protein}}$
	kg	*gm per cent*	*gm*		
Z10	1 2	1 82	9 5	199	0 047
Z24	0 9	0 89	4 3	182	0 024
Z14	0 6	0 59	2 7	135	0 020
T56	1 8	0 69	6 5	237	0 027
T67	0 7	0 81	3 4	129	0 026
Averages.	1 04	0 96	5 28	176	0 030

DISCUSSION

On the basis of the evidence presented (both our own observations as well as those of others) an hypothesis is offered that a relatively constant relationship (about 25 or 30 to 1) exists between dietary induced changes in the total tissue protein and changes in the total circulating serum albumin In other words, loss (or gain) of tissue protein and of serum albumin caused by diet parallel each other This conception is based on the existence of a fixed partition between tissue protein and serum albumin, *i e*, loss or gain in one is accompanied by a proportional loss or gain in the other Thus this hypothesis denies the existence of "deposit protein" or of reserves of protein which became available during dietary deficiencies (When loss of serum albumin occurs in other ways such as hemorrhage, burns, etc, another situation exists which presents other mechanisms)

It would seem that the existence of such a parallelism between serum albumin and tissue proteins makes it possible to detect the presence and extent of a tissue protein deficiency simply by determining the amount of serum albumin Since it is also probable that the same parallelism, or biological partition,

exists in the process of replenishment of depleted tissue protein and serum albumin, such information may be of practical value in calculating the amount of protein which must be fed in order to overcome the deficiency

If this idea is correct it is possible, as stated previously, to develop a formula by which the total protein loss may be calculated, if the serum albumin concentration is known Let the serum albumin tissue protein parallelism be represented by the equation $\frac{\text{Serum albumin depletion in grams}}{\text{Tissue protein depletion in grams}} = K$ The serum albumin depletion in grams will be equal to the normal serum albumin concentration (2) of dogs (3 6 gm.) minus the observed serum albumin concentration (S A) times the plasma volume (P V) When the albumin concentration is given in terms of grams per 100 cc. of serum, the plasma volume should be the number of 100 cc. of plasma present. This figure for the plasma volume is obtained by direct dye determination or estimated by dividing the normal body weight in kilos by 20 and multiplying this figure by 10 Stated again

$$\text{Serum albumin depletion (grams)}$$
$$= (3.6 - \text{observed albumin concentration S.A.})\ \frac{(\text{body weight in kilos} \times 10)}{20}$$

Inasmuch as K (Table I) is approximately 0 04, one obtains by substituting our first equation

$$\frac{(3\ 6 - \text{S.A.})\ (\text{body weight in kilos})\ (10)}{(20)\ (\text{tissue protein depletion in gm.})} = 0.04 \text{ or simplifying, tissue protein}$$
$$\text{depletion in gm} = (3\ 6 - \text{S A})\ (\text{normal body weight in kilos})\ (12.5)$$

The relationship of 1 to 25 (or 30, *i.e* $K = 0\ 04$ or 0 03) between serum albumin loss and tissue protein loss was compared with the available data on the relative amounts of each in the intact human body The amount of serum albumin is easily estimated as 161 gm (3500 × 4 6) Let us assume that nearly all tissue protein is muscle, from Vierordt (7) the weight of the muscles in an average adult is about 28 kilos of which 1/5 is protein or 5600 gm Of course only the parenchyma of the muscle tissue is lost during starvation so that the active protein tissue is less than this. Nevertheless 5600 gm. is but slightly more than 30 times the amount of serum albumin. It seems not unlikely that the relationship for K may be the same as the relative masses of these two protein compartments of the body

Certain obvious limitations of the hypothesis here formulated must be emphasized. It applies only when the tissue proteins and serum albumin are altered as a result of diet. It cannot be expected to hold in cases of hypoproteinemia due to blood loss, burns, nephrosis, or liver disease. Another factor is also important, *i.e.* in depleted patients the measurement of serum albumin concentration may not accurately measure the degree of hypoalbu-

minemia if the normal plasma volume shrinks For example, if the patient is dehydrated, the resultant hemoconcentration produces a rise in the value of serum albumin and minimizes or even masks completely the existence of the deficiency This masking effect of dehydration was pointed out clearly by Peters and his coworkers (8) in 1925 It is probable that the persistence of more or less normal plasma albumin concentrations, noted by several observers, in animals placed on certain low protein diets or fasted is the result of such hemoconcentration Indeed, this phenomenon was noted in experiments on fasting dogs performed in this laboratory (9)

Although the present data are confined to dogs, there is no reason to believe that a similar relationship does not hold true for human beings There are in the literature but few data of the sort herein discussed A rough calculation of the data of Bruckman, D'Esopo, and Peters (12) who measured the serum albumin concentration and made concomitant nitrogen balance studies of a malnourished individual during a period of replenishment indicates a value for K of about 0 02 Nevertheless if we assume that the value for K for humans is the same as for dogs, and accept as the normal serum albumin concentration the lower limit, 4 6 gm per cent, given by Hawk and Bergheim (10) the equation describing the protein depletion of a malnourished human is grams protein depletion = (4 6 − S.A) (normal body weight in kilos) (12 5)

The practical application of the conception herein presented concerns to a large extent the problem of replenishment of chronic protein deficiencies which are of frequent clinical occurrence For example, a patient whose body weight in health is 60 kilos and whose serum albumin is 3 6 gm per cent would be deficient by (4 6 − 3 6) (60) (12 5) = 756 gm protein If such a patient retained but half of the nitrogen consumed, he would require twice 756 gm or 1512 gm Thus a period of over 2 weeks on a diet providing 100 gm protein per day would be required to reach this amount To this must be added the requirements for endogenous nitrogen metabolism which would be at least 4 × 6 25 or 26 gm per day Viewed in this way the therapeutic problem presented by a protein-deficient patient is largely a quantitative one, only by giving a large amount of protein can it be solved This is particularly difficult when the nitrogen-containing nutriment must be given intravenously or parenterally or when but a short period of time is available for correction The immense quantities of protein required make the use of human plasma or serum transfusions for this purpose an altogether impractical procedure at least in severely depleted patients The most promising approach at present is the use of hydrolyzed protein of high biological value (6, 11)

SUMMARY

In five dogs placed on a protein-poor diet for 3 weeks, the decline in total circulating plasma albumin was a small (3 per cent) but relatively constant

part of the total nitrogen lost. These data together with observations on dogs by others indicate that there is a constant relationship or partition in the loss (or gain) between plasma albumin and total body protein induced by diet. Based on this relationship a formula is developed for estimating the degree of total tissue protein deficiency from the value of the serum albumin concentration alone. This formula would also apply to the replenishment of protein deficiencies of dietary origin and it indicates that for every gram of increase in serum albumin desired about 30 gm must be retained for increases in other proteins of the body

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STUDIES ON HYPOALBUMINEMIA PRODUCED BY PROTEIN DEFICIENT DIETS

II Rapid Correction of Hypoalbuminemia with an ad Libitum Meat Diet*

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The practical problem of restoring protein deficiency induced by diet has been studied experimentally by Weech (1) and his coworkers who found a definite regeneration (in the following order of efficacy) when beef serum, egg white, meat, liver, or casein was added to the diet of dogs For example, after a 3 week depletion period, these workers observed an almost complete return to normal when 5 gm per kilo per day of beef chuck was added to the diet for 10 days. This was about twice the rate of regeneration in other experiments in which half this amount of meat was given.

In surgical and other patients suffering from hypoproteinemia of dietary origin restoration of serum protein must often be achieved in a relatively short period of time. It has seemed important, therefore, to determine how fast the serum protein can be restored to normal when much larger amounts of protein are given. This was the purpose of the present experiment.

Methods

Five dogs were fasted for 3 weeks however, they were allowed water *ad libitum* and some of them were given Ringer's solution by gavage. At the end of 3 weeks they were started on the regeneration diet, which consisted solely of raw lean horse meat given *ad libitum* *i.e.*, sufficient meat was offered so that some of it was left at the end of each 24 hour period. This was then subtracted from the total to record the amount actually eaten.

Technical Methods —Hematocrits were determined on heparinized blood. Plasma proteins were then determined and fractionated by methods already described (2) The blood volume was determined by using the Evan's blue dye T 1284 according to a simplified technique as follows

The method for the determination of plasma volume in dogs was adapted from that described by Gibson and Evelyn (3) The main difference was the use of the Klett

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photoelectric colorimeter instead of the Evelyn apparatus employed by these workers Many of these details were worked out in the Department of Physiology of the Washington University Medical School, and the authors are indebted to Miss Rolf for her help in applying them to our purposes

An initial heparinized blood sample was first obtained Through the same needle a 200 mg per cent saline suspension of Evans blue was injected, the total dose being 1 mg of dye per kilo of body weight To insure the accurate introduction of the calculated amount of dye a 3 way-stop-cock and an 18 inch rubber tubing of small diameter connected with a syringe barrel was employed After the initial blood sample was drawn saline was allowed to flow by gravity from the syringe barrel into the dog's vein to test the position of the needle in the vein This assured, the dye solution was added by pipette under the surface of the saline solution Toward the end of the injection small portions of saline were added to wash the last remnants of dye from the tubing into the vein The initial time of mixing (zero point) was considered to be the time of the first saline washings

Samples of heparinized blood for dye estimation were taken subsequently at recorded intervals of approximately 15, 25, and 35 minutes from the initial (zero) time All blood samples were centrifuged for 30 minutes at 3000 R P M. 1 cc of plasma from each of the 4 samples was measured into test tubes and 5 cc. of saline added. After the contents of the tubes were mixed, readings were immediately made on the Klett photoelectric colorimeter using 620 and 540 filters, the two filters are used to correct for any hemolysis present in the dye samples The blank dye-free plasma was set at zero The correct R for each sample was calculated according to the formula which is nearly the same as that used by Gibson and Evelyn (3)

$$R = \frac{(R_{620}) \times 20 - (R_{540})}{19\ 5}$$

The corrected R values for each of the 3 samples were then plotted against time and a line was drawn through these points and carried back to zero

The concentration of dye (as mg per cc.) for the R at zero time was read off a previously prepared graph which established the relationship between R and the concentration of dye This graph was made by setting up several series of tubes each containing a known amount of dye and 1 cc of plasma, made up to 6 cc with saline. Because we found such dilute solutions of Evans blue to be unstable, a fresh 1 mg per cent saline solution was made from the 200 mg per cent dye solution each time a series was to be read A straight line always resulted at least in the range of dye concentration used in our experiments

The plasma volume in cubic centimeters was then calculated by dividing the milligrams of dye injected by the milligrams of dye present in 1 cc of plasma at zero time

FINDINGS AND COMMENTS

The main results of the experiment are recorded in Table I, which shows that after a 3 weeks fast the *ad libitum* meat diet resulted in a complete regeneration in 1 week of both the concentration of serum albumin and the total amount

thereof. Indeed the total circulating albumin increased well above normal, although this was undoubtedly a reflection of the increased plasma volume which accompanied the fall in red cell volume (hematocrit) There was very little further increase in the albumin during the 2nd week of the regeneration period. The fall in body weight and its subsequent rise, though a little more pronounced, roughly paralleled the behavior of the serum albumin The red cell volume decreased but slightly during the fast, during the regeneration period it fell markedly, a reflection of the tremendous increase in the plasma

TABLE I

Changes during a 3 Week Fast and during 2 Weeks of an ad Libitum Meat Diet

Dog	Initial				After 3 wks.					After 4 wks.					After 5 wks.				
	Wt.	Hem.	Alb	Glob.	Wt.	Hem.	Alb.	T A	Glob	Wt.	Hem.	Alb.	T A.	Glob	Wt.	Hem	Alb.	T A.	Glob
	kg	*per cent*	*gm. per cent*	*gm. per cent*	*kg*	*per cent*	*gm. per cent*	*gm*	*gm. per cent*	*kg*	*per cent*	*gm. per cent*	*gm*	*gm. per cent*	*kg*	*per cent*	*gm. per cent*	*gm.*	*gm. per cent*
E6	5 6	48 8	2 65	2.75	4.3	44 0	2 23	7.52	3 01	5 0	40 9	2.56	9 78	3 11	5.3	33.3	2 44	9 83	3 40
E2	7 4	46.7	3 64	2.78	5 4	50 4	3 07	8 96	2.56	7 1	39 6	3 54	15 72	2.44	7 4	32.5	3 42	17 41	2 70
F3	8.6	45 1	3.39	2 90	6 8	42 4	2 94	14.38	2 58	8 7	36 2	3 22	17 90	2.61	8 7	38 0	3.30	19.31	3 57
59	8 0	51 1	3 26	2 17	6 4	43 2	2 76	8 75	2.52	6 7	35 1	3 21	15 20	2.96	7 4	35 6	3.50	16 66	3 46
32	13 4	53.3	3 14	3 52	10 3	46 2	2 44	15 01	4 12	12 4	41 1	2.99	26 40	3.36	12 8	44 0	3 20	25 76	3 82
Averages																			
Wt.	8 6				6 8					8 0					8.3				
Hem.		49 0				45 2					38 6					36 7			
Alb			3 22				2 69					3 10					3 17		
T A.				13 9*				10 92					17 00					17 79	
Glob				2.82					2 96					3 00					3.39

* Estimated.

Abbreviations wt, body weight hem. hematocrit alb. plasma albumin glob., plasma globulin T A. total plasma albumin.

volume. The serum globulin, unchanged during the fast and for the 1st week of the regeneration period showed a pronounced increase in the 2nd week

The amount of meat ingested by the animals was surprisingly large and averaged 250 gm per kilo per day, *i.e.*, the dogs consumed one-fourth of their body weight each day Inasmuch as meat is composed of one-fifth protein the actual protein intake was 50 gm per kilo of body weight per day It was interesting to note that this large amount was eaten even during the last days of the experiment, *i.e* when body weight and serum protein had returned to normal.

These findings indicate first, the tremendous amount of protein which a depleted dog will ingest, and second, the rapidity with which such a high protein intake will lead to a complete correction of hypoproteinemia induced by a

3 weeks fast. The practical inference is obvious and points the way toward rapid relief of clinical hypoproteinemia by the administration of sufficient protein This is suggested in the accompanying study from this laboratory (2) in which a partition of about 30 to 1 was found in the distribution of dietary nitrogen loss or gain between tissue and serum proteins respectively, in other words evidence was presented showing that the body tissues will take up 30 gm of nitrogen for every gram which goes to the regeneration of serum protein. This explains why large amounts of protein are needed to correct hypoproteinemia The present findings are corroborative in that they indicate that if the protein intake is unusually large (in this case 50 gm per kilo per day) regeneration of serum proteins will be complete within a week We have no data as to whether this return to normal might not have occurred within a few days However, a continuation of the high protein intake, *i e* during the 2nd week did not raise the albumin level any further though for some reason it did instigate an increase of the globulin fraction during this last period

The failure of the red cells to regenerate as shown by the fall in the hematocrit is not surprising in view of the well known fact that restoration of red cells requires a number of weeks From the practical point of view this is not a serious deficiency inasmuch as the important clinical manifestations of protein depletion are concerned with the plasma and not with the erythrocyte portion of the blood The increase in the plasma volume during the regeneration period (from an average of 748 to 897 cc during the 4th week) was probably the reason for the particularly pronounced fall in the red cell volume during this time

SUMMARY

Serum albumin depletion, induced by a 3 weeks fast, was completely corrected within 1 week by the ingestion of a diet consisting solely of meat administered *ad libitum* The average protein intake was 50 gm per kilo of body weight per day A 2nd week of the same diet produced no further increase in serum albumin though it did lead to an increase in the globulin fraction

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THE LOCALIZED ACTION ON THE SPINAL CORD OF INTRA-MUSCULARLY INJECTED TETANUS TOXIN

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The pathogenesis of local tetanus has been the subject of much dispute. Until recently, the most generally accepted view was that of Marie and Morax (1902) and Meyer and Ransom (1902-03) They believed that local tetanus is the result of the action of tetanus toxin on the anterior horn cells of the spinal cord, the toxin reaching the central nervous system by way of the axons of the motor nerves On the other hand, Vaillard and Vincent (1891) and Zupnik (1905) held that local tetanus is the result of the action of the toxin directly on the muscle, and that the toxin reaches the central nervous system through the blood stream rather than along peripheral axons, causing there generalized rather than local tetanus

No one doubts the susceptibility of the central nervous system to tetanus toxin. If toxin is injected directly into the spinal cord in small amounts, a typical local tetanus may result (Fröhlich and Meyer, 1915) The question then remains whether local tetanus following intramuscular injection of small amounts of toxin is due to the action of the toxin on the segments of the spinal cord which innervate the site of injection, or on some peripheral structure If the former is true, it remains to be demonstrated how the toxin reaches the spinal cord.

In addition to the immunologic evidence for the nerve transport of tetanus toxin recently reviewed by Friedemann, Hollander and Tarlov (1941), certain other experiments have supported this theory Courmont and Doyon (1894) injected tetanus toxin into the right hind paw of a dog When the animal had developed stiffness limited to the right hind leg, they cut the spinal cord at the union of the lumbar and thoracic levels and sectioned all the dorsal roots. Stimulation of the dorsal roots with an inductorium was now effective in producing a reflex contraction with a current strength on the tetanic side one-fourth that necessary on the normal side. After removal of the spinal cord, stimulation of the ventral roots was effective at the same strength of stimulus on both sides. They therefore concluded that the action of the toxin is not on the peripheral motor elements of the reflex arc and must be either on the sensory nerves or on the spinal cord

An experiment reported by Sherrington (1905) further clarifies the situation

potential of A fibers following a single stimulus was equal on the right and left By increasing the amplification proportionally on the two sides, comparable records could be obtained at higher amplification

RESULTS

(a) *The Muscular Response*—The animal with well developed local tetanus exhibits a striking picture suggestive of reflex hyperactivity A weak stimulus to the affected limb produces a vigorous increase in the extension of the limb This type of response might depend upon any or all of a variety of factors For example, long lasting repetitive responses of the nerves, myoneural junctions, or muscles, to single stimuli, could account for it, independent of any change in the spinal mechanisms The experiments of this and the succeeding section were designed to test this possibility

The augmented response of the tetanic leg to a weak stimulus, described above, was studied in a series of cats by recording the electric and mechanical responses of the calf muscles The stimulus used was either a sudden tap on the Achilles tendon or a shock to the sciatic nerve The difference between the tetanic and the control legs was apparent, as can be seen in Fig 1, where A and B represent the tetanic side and D and E represent the control The increased mechanical response of the tetanic leg is accompanied by a fusillade of muscle action potentials

If this augmented response depended upon an altered response of the motor nerve or of the muscle, the difference between the sides should persist after section of the sciatic nerve central to the stimulating electrodes As may be seen in Fig 1, C and F, when this was done the electromyograms of the two sides were indistinguishable and the mechanograms differed only in amplitude

The augmented response of the leg with local tetanus does not depend, therefore, upon a change at the myoneural junction, in the muscle, or in the motor nerve This is in agreement with the experiments of Courmont and Doyon (1894) and others Repetitive responses of the muscle to single stimuli applied to the cut motor nerve, as described by Harvey (1939), were not observed by us Differences in technique may account for the differences in result Although our experiments do not exclude changes in the muscle due to tetanus toxin, muscular changes do not explain the phenomena described

(b) *The Peripheral Nerve*—The following experiments demonstrate that the augmented response after a single excitation of the sciatic nerve, described above, is not due to repetitive activity of either the motor or the sensory components of the nerve trunk

After section of the posterior tibial and peroneal nerves in the popliteal space, recording electrodes were placed on the branch of the posterior tibial nerve which innervates the lateral head of the gastrocnemius muscle, the portion of the nerve under the distal electrodes was crushed in order to secure

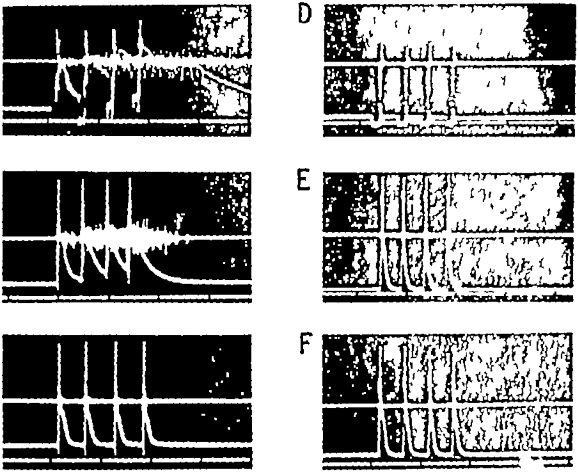

FIG 1 The augmented response of the tetanic leg and its dependence [illegible] arc. Mechanical and electric records from the calf muscles of a cat with [illegible] local tetanus 4 days after injection of 1 133 cat M L D of toxin The spinal [illegible] had been sectioned at the level of the 12th thoracic vertebra 5 hours before the records were taken. In each record the uppermost line is the electrogram taken from needle electrodes, one in the belly of the muscles the other at the ankle The second line is the mechanogram obtained with an optical frictionless torsion spring myograph The peak of each contraction curve obscures part of the corresponding initial electrogram The bottom line is a time signal marking intervals of 1 second

A Responses on the tetanic side to taps on the Achilles tendon

B Responses on the tetanic side to brief condenser discharges applied to the intact sciatic nerve at the hip

C Responses on the tetanic side as in B after section of the sciatic nerve central to the stimulating electrodes.

D E and F The same as A B and C respectively on the unaffected side

monophasic records. Stimulating electrodes were placed on the sciatic nerve at the hip The nerve was cut central to the stimulating electrodes and excited with maximal brief condenser discharges

Of 14 animals studied, 10 showed no repetitive response Repetitive re

sponses of equal degree were present on both sides in one instance, in two others there was a repetitive response limited to the side with local tetanus, and in one, there was a repetitive response on each side, but predominantly on the side with local tetanus

The excitability of the branch of the posterior tibial nerve to the lateral head of the gastrocnemius muscle was studied *in situ* in 8 animals with the preparation described above Strength-duration curves were determined by the method of Hill (1936) There was no significant variation in either the rheobase or the time constant between the nerve of the tetanic and that of the unaffected leg

The amplitudes of the maximal action potential of the A fibers of the nerve to the gastrocnemius muscle of the tetanic and control sides were compared in 22 animals In 12, the spike amplitude of the tetanic side exceeded that of the control, in 6 there was less than 10 per cent difference between the two sides, and in 4, the spike of the control exceeded that of the tetanic side Differences in the degree of shunt around the nerve and recording electrodes may account in part for the difference in the spike amplitude observed

Thus occasionally there may be changes in the peripheral nerve in local tetanus Yet the typical responses of local tetanus were present in many experiments in which repetitive response to single stimuli did not occur in the peripheral nerves In every instance in which repetition was demonstrated in the peripheral nerve by the technique outlined above, the presence of the abnormal response was obvious before the special tests were made In order to avoid confusion of peripheral and central effects, the experiments of the succeeding sections relate to animals in which there was electrographic evidence that the sciatic nerve, when severed from the central nervous system, did not respond repetitively to single stimuli

(*c*) *Changes in the Spinal Cord*—Since the augmented activity of the calf muscles associated with local tetanus is, as demonstrated above, independent of changes in the muscle, neuromuscular junction, and peripheral nerve, its origin must by exclusion be either in the dorsal root ganglia or in the spinal cord The following experiments were devised to study these possibilities

The spinal cord of 18 cats with local tetanus limited to one hind leg was sectioned either at the first, or more frequently the 12th thoracic level Stimulating electrodes were placed on the sciatic nerves at the hip, and the peroneal and posterior tibial nerves, the nerves to the biceps femoris and hamstring muscles, and in some instances the femoral nerves were sectioned Recording electrodes were placed on the central end of the divided branch of the posterior tibial nerve which innervates the lateral head of the gastrocnemius muscle, the portion of the nerve under the distal electrode was crushed in order to secure monophasic records

The electrograms obtained from this preparation on stimulating the ipsi-

lateral sciatic nerve differ strikingly on the tetanic as compared to the control side A single strong condenser discharge is followed by 3 negative waves The 1st of these is similar on the two sides and represents the volley of impulses initiated by the stimulus at the hip and descending the nerve The 2nd wave begins 3.5 to 4 milliseconds after the stimulus. Since it is abolished by section of the sciatic nerve central to the stimulating electrodes it may be called the early reflex response This wave is small in comparison to the amplitude of the 1st wave resulting from maximal activity of the A fibers of the nerve. If the strength of stimulus is increased from subthreshold to supramaximal values by steps, the early reflex response undergoes a series of changes it increases in amplitude until it reaches a maximum at a strength of stimulus still submaximal as judged by the amplitude of the 1st wave and then decreases with stronger stimuli The early reflex response resembles the *s* wave described by Renshaw (1940) in the reflex responses from ventral roots

If the sciatic nerve is stimulated repetitively at a frequency of 4 to 8 per second, the early reflex response undergoes a decrease in amplitude which may be interpreted as either fatigue or inhibition This decrease is regularly less pronounced on the side which is subject to local tetanus than on the control side Fig 2 illustrates this difference In other respects the early reflex response is similar on the two sides

About 7 to 11 milliseconds after the stimulus a 3rd wave occurs Like the 2nd wave, this disappears on section of the sciatic nerve central to the stimulating electrodes and hence may be called the late reflex response It is irregular in form on both the tetanic and the control sides but more strikingly so on the tetanic It probably represents the fusion of the responses of several groups of neurons firing asynchronously over a period of 6 to 16 milliseconds This wave starts earlier, lasts longer and is usually of greater amplitude on the tetanic than on the normal side, as may be seen in Fig 3 Exceptionally the amplitude of the late reflex response of the tetanic side is no greater than that of the control

If the sciatic nerve is stimulated at the rate of 4 to 8 shocks per second the amplitude of the late reflex response on the control side either diminishes or remains unchanged The same procedure on the tetanic side usually leads to an increase in the amplitude of the late reflex response The latency of this response usually shortens with repetitive stimulation almost invariably more on the tetanic than on the control side (Fig 3)

If the response is examined with high amplification and with the coupling condensers of the amplifiers arranged most favorably to record activity of high frequency considerably more after-discharge is seen to follow the 3 waves described on the tetanic side than on the control Exceptionally the two sides showed no difference in this respect Repetitive stimulation at 4 to 8 shocks per second invariably results in an increase of the amplitude

and often of the duration of the after-discharge on the tetanic side, which may last as long as 3 seconds after the final stimulus This is in sharp con-

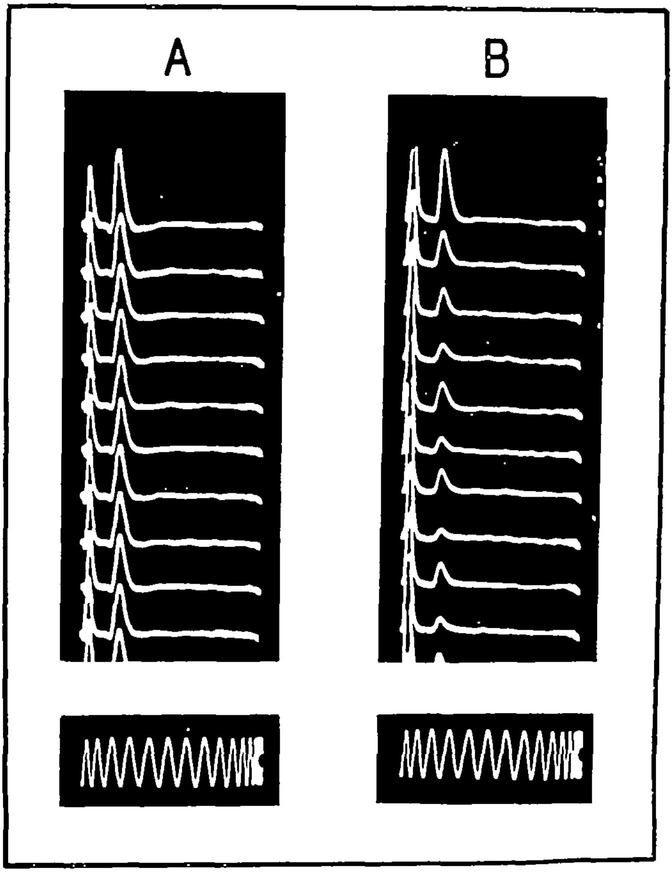

FIG 2 Greater decrease of the early reflex response to repetitive stimulation on the control side Electrograms from the nerve supplying the lateral head of the gastrocnemius muscle, 3 days after injection of 1/100 cat M L D of tetanus toxin into the right calf, in a cat in which the development of symptoms of local tetanus was prevented by extensive denervation of the legs 19 hours after the injection

A Response of the nerves of the tetanic side to 8 stimuli per second to the sciatic nerve at an intensity which produces a maximal amplitude of the 2nd wave

B The same as A on the unaffected side

trast to the electrogram of the control leg, in which summation is uncommon and never as great as may be seen in the injected leg Fig 4 illustrates the after-discharge on the two sides of an animal with local tetanus

Tetanus toxin injected into the calf muscles therefore produces a clearly

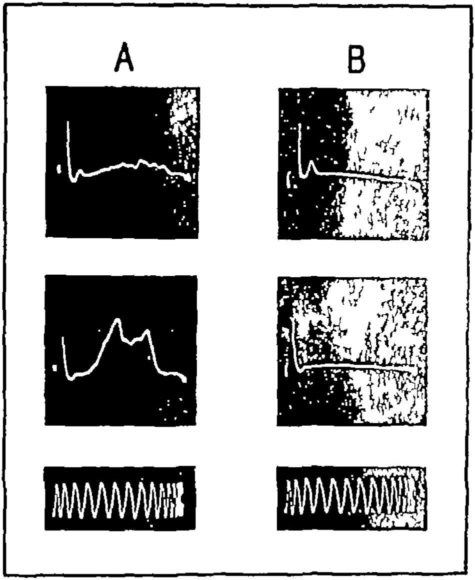

FIG 3 Differences in the late reflex response of tetanic and control leg Electrograms from the nerve supplying the lateral head of the gastrocnemius muscle 4 days after injection of 1/100 cat M.L.D of tetanus toxin into the right calf in a cat in which the development of symptoms of local tetanus was prevented by extensive denervation of the legs 18 hours after the injection

A Upper picture response on the tetanic side to a single strong stimulus to the sciatic nerve. The 3 waves described in the text are shown from left to right Middle picture response to the last of 8 stimuli delivered within 1 second showing summation and change of latency of the late reflex response and decline of the early reflex response Lower picture time signal each cycle represents 2 milliseconds

B The same as A on the unaffected side There is no summation in the late reflex response and the decline of the early reflex response is more marked than on the tetanic side

recognizable alteration in the pattern of nerve impulses which are discharged by the spinal cord in response to a stimulus applied to the sciatic nerve.

(*d*) *Effect of Section of Dorsal Roots on Electrogram of Nerve*—Three further

possible mechanisms might explain the augmented response of the tetanic leg It might be due to an altered state of the spinal cord itself, or of the dorsal root ganglion, or to a change in the activity of peripheral sensory mechanisms The latter two possibilities were tested in the following experi-

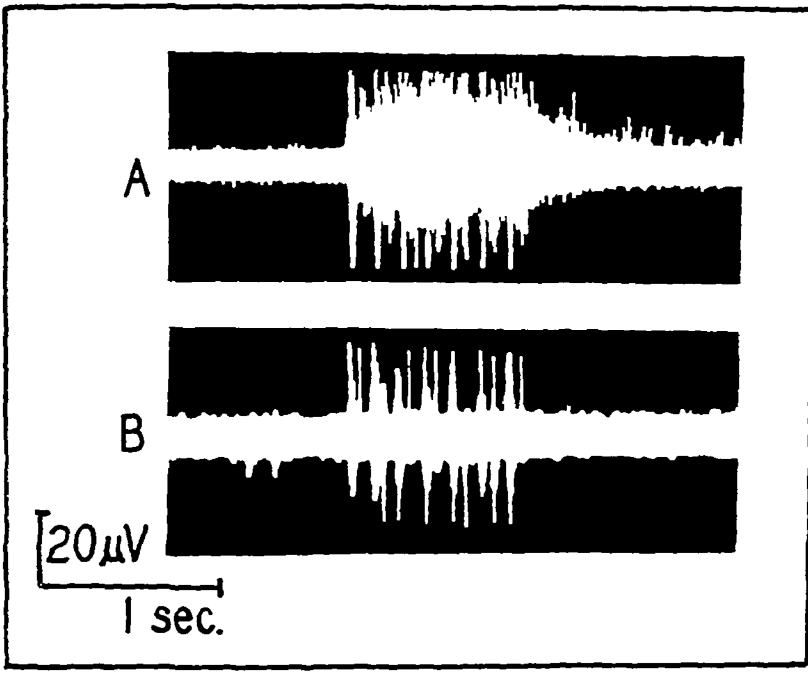

FIG 4 Augmented after-discharge on the tetanic side Electrograms from the nerve which innervates the lateral head of the gastrocnemius muscle, following stimulation of the 7th lumbar dorsal root, in a cat with unilateral local tetanus 9 days after injection of 1/75 M L D of tetanus toxin The spinal cord had been sectioned at the level of the 12th thoracic vertebra 5 hours before the records were taken, and all the dorsal roots below this level cut bilaterally These records were taken at high amplification, with the coupling condensers arranged to emphasize asynchronous discharge of the nerve

A Electrogram of the affected side upon repetitive stimulation of the sciatic nerve

B The same as A on the unaffected side

ments by complete deafferentation of the spinal cord by section of the dorsal roots central to the dorsal root ganglia

The effect of section of the dorsal roots was studied in 11 cats with tetanus limited to one leg The spinal cord was sectioned under ether anesthesia at the level of the 12th thoracic vertebra, and the lamina of the lumbar vertebrae

removed The animal was then allowed to recover from the effects of the anesthesia, and was symmetrically fixed as in earlier experiments Stimulating electrodes were then placed bilaterally upon the sciatic nerve and recording electrodes on the nerve to the lateral head of the gastrocnemius muscle In each case the presence of tetanus limited to one leg was confirmed by record

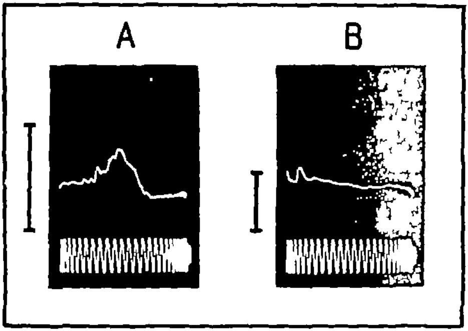

FIG 5 Reflex response to dorsal root stimulation Electrograms from the nerve which innervates the lateral head of the gastrocnemius muscle following stimulation of the 6th lumbar dorsal root in a cat with unilateral local tetanus 7 da after injection of 1/75 cat M L D of toxin Five hours before the records were taken the spinal cord was cut at the level of the 12th thoracic vertebra All the dorsal roots below this level were sectioned bilaterally The vertical lines at the left of the pictures represent the relative heights of the maximal spikes of the A fibers of the respective nerves the amplitude of the response of the unaffected side must therefore be doubled to compare it with that of the tetanic side.

A. Upper line electrogram from the nerve of the injected leg after a single strong stimulus to the dorsal root Lower line time signal each cycle represents 2 milliseconds.

B The same as A on the unaffected side. In this experiment the early reflex response was greater on the control than on the tetanic side The late reflex response showed the usual predominance on the tetanic side

ing the electric response of the nerve supplying the gastrocnemius on stimulation of the sciatic nerve Four or 5 hours after the section of the spinal cord the dura mater was laid open and the dorsal roots of the lumbosacral segments cut central to the dorsal root ganglia The cauda equina was sectioned at the upper border of the sacrum The dorsal roots were then stimulated through silver wire shielded electrodes stimulation at the 7th lumbar level was usually most effective

In 7 of 11 experiments, the condition of the spinal cord after this procedure seemed satisfactory as judged by the responses elicited by stimulating the dorsal roots In 2 of these 7, the responses of the left and right sides were equal In the remaining 5, the electrogram of the tetanic side showed the changes typical of local tetanus as outlined in Section (*c*) A single strong condenser shock evoked 2 negative waves, corresponding to the 2nd and 3rd waves, the reflex responses, seen following stimulation of the sciatic nerve In all 5 experiments, repetitive stimulation of the dorsal root of the tetanic side at the rate of 4 to 8 shocks per second resulted in little or no decline of the early reflex response, and an increase in the amplitude of the late reflex response When the corresponding dorsal root of the control side was stimulated, the initial magnitude of the response was smaller (Fig 5), and after repeated stimulation there was either no change or a decrease in the magnitude of the late response In 4 of these 5 animals, repetitive stimulation of the dorsal root elicited an after-discharge lasting about 0 4 second on the injected side when examined with high amplification as in earlier experiments (Fig 4) An after-discharge was never seen in the nerves of the control side under similar conditions

The origin of the augmented activity of the tetanic gastrocnemius muscle after stimulation of the sciatic nerve is therefore within the spinal cord, and limited to the same side as the local tetanus

(*e*) *Effect of Denervation after Injection of Toxin on the Development of Local Tetanus in the Spinal Cord*—It is conceivable that the changes in the spinal cord described above are dependent on changes in the peripheral structures For example, changes in the muscle following intramuscular injection of tetanus toxin might result in a prolonged continuous bombardment of the spinal cord by afferent impulses which might produce the observed changes This possibility can be excluded by sectioning the nerves of the injected area before the appearance of local tetanus

After the injection of 1/100 cat M L D of tetanus toxin into the gastrocnemius muscle neither the physical signs nor changes in the electrogram of nerve characteristic of local tetanus are present within the first 24 hours The right calf muscles of 3 animals were injected with 1/100 cat M L D of tetanus toxin Eighteen to 20 hours later, at a time when none of these animals showed physical signs of tetanus, the following operations were performed bilaterally under ether anesthesia The femoral and obturator nerves were cut in the groin, and the nerve to the hamstring muscles at the hip The popliteal space was cleared of fat, and all the branches of the peroneopopliteal nerve were cut—namely, the nerve to the biceps femoris muscle, and the peroneal, posterior tibial, and sural nerves For later identification, a black silk suture was tied to the central end of the cut branch of the posterior tibial nerve which innervates the lateral head of the gastrocnemius muscle Three, 4, and 5 days

after injection, when control cats injected at the same time with the same amount of toxin exhibited typical local tetanus, there were no signs of tetanus in the animals upon which operations had been performed The hips, knees, and ankles of the latter were flaccid, and both upper legs were held abducted Under ether anesthesia the spinal cord was cut at the level of the 12th thoracic vertebra, after which the animals were permitted to recover from the effects of the anesthesia. On each side, stimulating electrodes were placed on the sciatic nerve and recording electrodes on the branch of the posterior tibial supplying the lateral head of the gastrocnemius The pattern of the electrogram of the nerve to the gastrocnemius muscle of the injected leg agrees in all respects with that previously described for the nerve when the muscular picture of local tetanus is present. The electrogram of the control side is likewise the same as that of the uninjected leg in animals in which the innervation is intact. Figs 2 and 3, which have been used to illustrate the characteristic waves, are from animals in which the leg had been denervated

In local tetanus, therefore, changes develop in the spinal cord even though section of the peripheral nerves 18 to 20 hours after injection of the toxin has prevented the appearance of the peripheral signs of local tetanus

(*f*) *Effect of Denervation before Injection of Toxin on the Development of Local Tetanus in the Spinal Cord*—The localization of the spinal effects of intramuscularly injected toxin to the region supplying the innervation of the injected area is difficult to explain unless the toxin reaches the cord by of way the nerve trunks In this case section of the nerve trunks supplying the area of injection before the injection of the toxin should prevent the spinal effects. The following experiments were devised to test this.

The procedure was the same as that described in the preceding section except that the section of nerve trunks was performed shortly before the injection of the toxin. Three animals so prepared received 1/100 cat M.L.D of tetanus toxin into the right calf muscles Three, 4, and 5 days later none of the animals showed signs of local tetanus The spinal cord was cut under ether anesthesia and the electrograms from the nerves supplying the lateral head of the gastrocnemius were studied as in the previous sections None of the changes characteristic of local tetanus was present on either side in these animals.

The localized action of tetanus toxin on the spinal cord when the toxin is injected into the calf muscles can therefore be prevented by cutting the nervous pathways which connect the area of injection with the cord.

(*g*) *Effect of Local Trauma on the Localization of Tetanus*—It has been suggested that the tetanus toxin reaches the central nervous system through the circulation, but that the trauma of injection determines its localization to that part of the spinal cord from which the innervation of the site of injection arises. In 5 cats, 0 4 cc of tetanus toxin which had been inactivated by boiling was

injected into the left calf muscles at the same time that active toxin was injected into the muscles of the right calf In no instance did signs of local tetanus appear in the left leg, nor did oscillographic records from the nerves of the left leg 2 to 4 days after injection reveal the changes characteristic of local tetanus The trauma of injection, therefore, played no rôle in the localization of the tetanus Gumprecht (1895) excluded this possibility in a more drastic fashion He injected tetanus toxin into the right hind leg and croton oil into the left foreleg of mice Local tetanus appeared in the former but not the latter leg

DISCUSSION

The experiments reported in Sections (*a*) to (*d*) demonstrate that local tetanus in the cat resulting from the intramuscular injection of toxin is associated with a characteristic response originating in that region of the spinal cord from which the innervation of the injected area arises The experiments of Section (*e*), in which the nerves of the legs were sectioned after the injection of toxin but before the appearance of any signs of tetanus demonstrate further that the change in the spinal cord is not dependent upon changes in the muscle or neuromuscular junction but represents an action of the tetanus toxin on the spinal cord itself

How, then, does the toxin injected into the leg reach the corresponding segment of the cord? If it were carried from the site of injection to the spinal cord only through the blood stream, it is difficult to understand why its action is limited to this small segment With the doses of toxin used the contralateral portion of the spinal cord at the same level remains unaffected The localization of tetanus is more understandable if the toxin be supposed to ascend the peripheral nerves to the spinal cord This view is supported by the experiments of Section (*f*), in which the action of the intramuscularly injected toxin on the spinal cord was prevented by section of the nerves of the leg before the injection of the toxin This theory of the path of transport, as well as the logical steps leading up to it, were probably first proposed by Gumprecht (1894, 1895) The present observations establish facts which are adequately explained by this theory and by none other yet advanced It may be concluded that in local tetanus the toxin is carried to the spinal cord by way of peripheral nerves

The experiments reported give no hint as to which element of the peripheral nerve may be the pathway of the toxin to the central nervous system Gumprecht believed that the tissue spaces of Key and Retzius were the route Marie and Morax (1902) and Meyer and Ransom (1902) suggested that the ascent was through the axis cylinder itself

Abel (1934, 1935, 1938) argued that the peripheral nerves cannot serve as pathways for diffusable substances to the central nervous system He questioned the technique of investigators who had reported the passage of dyes

up nerves to the spinal canal Perdrau (1937), however, used techniques free from the objections raised by Abel, and confirmed the previous results He demonstrated that the central end of the cut sciatic nerve of anesthetized cats is capable of absorbing dyes from solutions into which the nerves are dipped, and that these dyes diffuse up the axis cylinder as far as the spinal cord at a rate sometimes as fast as a centimeter per hour Perdrau pointed out that the passage of substances through the axis cylinder is compatible with de Renyi's observation that the consistency of the axis cylinder is that of a soft jelly

The suggestion that tetanus toxin reaches the central nervous system by way of the peripheral nerves is not unique Strong evidence indicates that the viruses of the poliomyelitis (Bodian and Howe, 1941) and of rabies (di Vestea and Zagari, 1889), for example, reach the central nervous system in this way

SUMMARY

Local tetanus limited to one leg was studied in cats after intramuscular injection of tetanus toxin

1 The electric and mechanical response of the affected muscle after a single stimulus to the intact sensory motor nerve is greater in amplitude and duration than the response of the corresponding muscle of the unaffected leg (Fig 1)

2. This augmented response of the muscle is associated with an augmented response arising from the ipsilateral portion of the spinal cord, while the contralateral part of the cord is unaffected, as demonstrated by electrographic records from the motor nerves (Figs 2 to 5)

3 The augmented muscular response is abolished when the reflex arc is broken, but the augmented response in the spinal cord is independent of changes in the muscle, the neuromuscular junction, the afferent and efferent peripheral nerves, and the dorsal root ganglia.

4 The augmented spinal response develops in the absence of the peripheral signs of local tetanus. Hence the pathogenesis of the altered state in the spinal cord is independent of the peripheral effects of the toxin.

5 In local tetanus, therefore, the toxin injected intramuscularly acts selectively upon the segments of the spinal cord which supply the innervation of the injected area.

6. The augmented spinal response may be prevented by section of the nerve trunks supplying the area of injection prior to the injection of the toxin.

7 It is concluded that in local tetanus the toxin is carried to the spinal cord by way of peripheral nerves.

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COPPER AND COBALT
RELATED HEMOGLOBIN PRODUCTION IN EXPERIMENTAL ANEMIA*

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Copper has been shown to have a definite effect in anemias due to a milk diet (11) and we have indicated a probable effect in experimental anemia due to blood loss (12, 4) The experiments below show the effect of various doses of copper alone and combined with the standard dose of iron The hemoglobin production is somewhat irregular, suggesting that larger doses of copper may at times inhibit hemoglobin production.

Cobalt has been used by various investigators especially during the past ten years. Some claim that cobalt stimulates hemoglobin production (10) or that polycythemia can be produced in various animals by cobalt feeding (14, 3) Others find no definite evidence that cobalt influences hemoglobin production (1) Many of these conclusions are reviewed in a recent paper by Frost, Spitzer, Elvehjem, and Hart (6)

Among other difficulties, a definition of polycythemia in dogs is not satisfactory The polycythemia values given in many papers for dogs come within the range which we consider normal. The dogs used in this laboratory for years to test hemoglobin production in anemia due to blood loss are bred and raised in our kennels. Not of a pure strain, they are pretty uniform as to type—the white bull terrier strain predominating These adult dogs when considered to be perfectly normal on a mixed diet present a 50 to 55 per cent hematocrit, 19 to 20 gm. hemoglobin per 100 cc. and 7 to 9 million red cells. If one takes a dog with 15 gm. hemoglobin and raises the level to 20 gm. hemoglobin it does not convince us that this is a true polycythemia for the dog. Perhaps diet, environment and inheritance all come into the picture but short term experiments do not seem significant. We have no opinion about animals other than dogs from actual experience but we feel that short periods during which the red cell and hemoglobin rise above short "normal" periods do not constitute polycythemia as we understand it.

Methods

The production of this experimental anemia in otherwise normal dogs by bleeding and the routine care of these animals has been described (15) The anemia is continuous usually from maturity (1 year of age) and the basal ration of salmon bread

* We are indebted to Eli Lilly and Company for aid in conducting this work.

(a complete diet) is fed during all control periods The anemia level is 6 to 7 gm. hemoglobin and is reached by careful bleeding over 12 to 14 weeks—the normal hemoglobin level being 19 to 20 gm. In this way all reserve stores of hemoglobin building materials are exhausted. These dogs are more susceptible to various infections and are protected by isolation and by vaccination against distemper and dysentery—kept in air conditioned rooms with temperature control Clinically they are active and normal with excellent appetites at the anemia level which is continued throughout a normal life—8 to 11 years

The tables below present only the net hemoglobin output although the red and white cell counts, blood volume, and hematocrit are followed and recorded weekly in the histories The *net hemoglobin output* is obtained very simply by adding together all the hemoglobin removed during the 2 week feeding (copper, iron, cobalt) period plus the following 3 weeks, less the control basal ration hemoglobin production—usually 2 to 3 gm per week The week following the metal feeding often shows a high hemoglobin output—a carry over which is exhausted during the 2 weeks after period

The metals are given as various salts but the figures in various tables refer to the metal alone

EXPERIMENTAL OBSERVATIONS

There is a tendency to explain responses like hemoglobin production in anemia as due to an inflow of a substance which has been depleted and therefore lacking for new hemoglobin production The *iron* effect seems to be a true response to an essential substance which is lacking and required to form new hemoglobin in anemia *Copper* on the contrary is not lacking but is present more abundantly in the liver of the anemic dog than in the normal dog Yet copper feeding in modest amounts often does cause an increase in hemoglobin production in these experiments (Table 1) *Amino acids* when fed may likewise cause an increase in hemoglobin production in similar experiments (16) In these dogs there is no lack of protein stores out of which the dog can make plasma protein (9) Moreover, the fasting, anemic dog given abundant iron can produce 100 gm new hemoglobin or more (2) Therefore, we cannot safely assume that this response is due to a *lack* of the given amino acid but rather that the dog can use various amino acids to good purpose in its internal protein metabolism We prefer to look at these responses to the feeding of copper or amino acids as indications of a change in flow of the protein precursors into the hemoglobin channel—whether this change is associated with enzyme systems or other mechanisms

Table 1 presents a considerable number of experiments in which various amounts of copper sulfate and tartrate are added to the basal salmon bread ration This bread contains iron (1.5 mg per 100 gm) as fed The average daily intake is 300 to 400 gm salmon bread There may be a response to as

little as 5 mg. copper daily. The most uniform response is noted in the experiments with 10 to 15 mg. copper daily, an average value of 22 gm. hemoglobin output per 2 weeks feeding. With larger doses of copper the responses become more irregular and somewhat less in amount. Responses of less than

TABLE 1

Copper Added to Basal Ration (Salmon Bread)

Hemoglobin net output for 2 weeks feeding

Dog. No.	Weight	Copper daily dose	Hb output due to Cu	Control hemoglobin production		
				Fe 40 mg. daily	Liver 300 gm. daily	Salmon bread ration
	kg.	mg.	gm.	gm.	gm.	gm.
34-3	15	5	0	52	76	4
32-5	16	5	3	54	97	4
37 23	16	5	54	60	71	4
25-23	14	5	26	50±	82	4
35-2	22	10	26	84	99	8
37 20*	19	10	14	23	46	4
37-83*	14	10	14	59	67	4
27 238	16	13	38	53	—	14
37 23	21	15	15	58	91	16
37 23	19	15	30	60	71	4
32-5	16	15	21	53	112	14
36-12	22	15	18	59	82	4
37 21	18	20	30	61	107	4
30-117	18	20	36	53	92	4
35-4	17	20	5	55‡	85	4
40-26	13	20	0	44‡	95	4
29-326	18	30	6	74	86	4
32 5	16	30	9	54‡	97	4
39-2	18	30	18	44‡	103	4
27 241	14	40	20	43	118	14
37-87	19	40	9	61‡	72	16
37 22	21	40	5	46‡	73	4
37 23	17	40	5	69‡	74	6
30-117	17	40	7	53	92	4

* Bile fistula.

‡ Iron feeding immediately followed the copper feeding

10 gm. hemoglobin we class as negative. Frequently a copper feeding of 30 to 40 mg. daily will give a negative response but subsequently a surplus of hemoglobin may be removed over and above the base line output. This suggests an initial inhibition with subsequent over production in a long continued bread feeding after period. We have observed somewhat similar responses to infection in these anemic dogs—an initial depression followed by periods of over production of hemoglobin (13)

The control hemoglobin production due to salmon bread is about 4 gm per 2 weeks when the iron content of the salmon bread is kept at a minimum of 1 5 mg iron per 100 gm The higher values recorded in Table 1 (8 to 16 gm hemoglobin) are due to bread containing a higher level of iron—an iron effect in other words

TABLE 2

Copper and Iron Given Together

Hemoglobin net output for 2 weeks feeding

Dog No	Weight	Daily combined dose		Hb output due to Cu + Fe	Control hemoglobin production			Remarks
		Cu +	Fe		Fe 40 mg daily	Liver 300 gm daily	Salmon bread ration	
	kg	*mg*	*mg*	*gm*	*gm*	*gm*	*gm*	
27-238	15	2	21	36	48	—	4	
27-236	17	4	40	73	57	86	10	
27-234	17	4	40	67	72	108	10	
23 1	18	4	40	64	46	107	14	
37-20	17	5	40	18	27	62	4	Bile fistula
34-146	17	5	40	61	49	82	4	Eck fistula
34-146	18	5	400	65	54	82	4	Repeat experiment
29-326	16	5	40	52	48	67	4	
37-20	16	10	40	44	23	62	4	Bile fistula
35-2	22	10	40	84	97	99	8	
34-145	20	10	40	59	54	90	22	
37-83	14	10	40	39	59	67	4	Bile fistula
37-82	17	15	40	55	47*	75	4	
37-23	20	15	40	62	58*	91	8	
35-4	18	20	13	63	48	85	4	
37-21	18	20	40	22	61	73	4	
37-21	17	20	40	78	61	73	4	Repeat experiment
32-5	17	30	40	26	34*	97	20	
37-87	20	40	40	49	56*	72	16	
27-241	14	40	40	67	43	118	20	
29-65	13	40	40	75	59	103	10	
36-12	21	40	40	75	70	85	4	

* Iron feeding immediately followed copper and iron feeding

Table 2 shows *in general* the negative effect of adding copper to standard doses of iron The hemoglobin production due to iron alone is very like that due to the same iron dose combined with various amounts of copper The bile fistula dog presents a subnormal output of hemoglobin due to various food factors including iron (8) Copper causes no unusual effect in the bile fistula dog The experiments with the Eck fistula (dog 34-146, Table 2) show nothing unexpected The Eck fistula dog may at times present a subnormal hemo-

globin production in standard anemia experiments The intake of 400 mg iron daily usually raises the hemoglobin output to the level of the liver feeding (dog 34-146) Occasionally a dog will present responses which are subnormal

TABLE 3

Cobalt Added to Basal Ration (Salmon Bread)

Hemoglobin net output for 2 weeks feeding

Dog. No.	Weight	Cobalt daily dose	Hb output due to cobalt	Control hemoglobin production			
				Fe 40 mg daily		Liver 300 gm. daily	Salmon bread ration
				Before Co	After Co		
	kg	mg	gm.	gm.	gm.	gm.	gm.
34-3	15	5	5	52	34	76	4
34-87	20	10	0	56	90	72	4
37-85	19	10	10	75	55	72	4
32-5	17	20	0	54	111	97	4
37 22	23	20	5	74	71	73	4
30-114	15	25	0	56	—	94	18
36-12	22	30	0	59	70	82	4
35-4	20	40	9	57	61	84	4
37-87	20	40	0	56	40	72	16

TABLE 4

Cobalt and Iron Given Together

Hemoglobin net output for 2 weeks feeding.

Dog. No.	Weight	Daily combined dose		Hb output due to Co + Fe	Control Hb output			
		Co +	Fe		Fe 40 mg. daily		Liver 300 gm. daily	Salmon bread ration
					Before Co	After Co		
	kg.	mg.	mg.	gm.	gm.	gm.	gm.	gm.
29-326	16	10	40	54	50	—	67	4
37-85	19	10	40	31	55	73	72	4
37 22	22	20	40	60	74	56	73	4
35-2	21	20	40	53	49	61	116	8
37 21	16	30	40	26	61	50	75	4
39-1	16	30	40	46	47	44	63	4
39-2	19	40	40	23	44	34	103	4

and this condition usually continues throughout the entire anemic history of the animal (dog 29-326, Table 2) Repeat experiments (dog 37-21, Table 2) indicate that copper may at times modify the iron response in anemia, the first experiment showing a subnormal response and the second experiment showing

a high output of hemoglobin The experiment with dog 35-4 (iron 13 mg daily given with 20 mg copper, Table 2) shows a high response and the control hemoglobin output due to iron 13 mg daily was only 22 gm hemoglobin—compare response in same dog to 40 mg iron of 48 gm hemoglobin

Table 3 shows a number of experiments in which various amounts of cobalt are added to the basal ration The cobalt effect is completely negative in contrast to the copper effect (Table 1) When we compare the response to iron feeding tested before and after the cobalt feeding, we note that the iron response is more irregular after the cobalt experiment in contrast to the iron response before the cobalt The reason for this irregular response is not clear but there is little doubt that cobalt is slightly toxic to dogs

Table 4 shows clearly that cobalt added to the usual iron dose does not favor hemoglobin production On the contrary there appears to be a definite inhibition which can scarcely be due to anything but the cobalt This inhibitory effect may last over some weeks in certain dogs—for example, dog 39-2, Table 4, given cobalt 40 mg plus iron 40 mg shows an output of hemoglobin of only 23 gm in contrast to iron alone of 44 gm just before the cobalt feeding Immediately following the cobalt experiment, the standard dose of iron produced 34 gm hemoglobin and immediately following that, the same dose of iron showed an output of 48 gm hemoglobin—that is a late return to normal One may choose to explain these responses as due to cobalt toxicity

DISCUSSION

Copper at times has a definitely favorable effect upon hemoglobin production in standard anemic dogs—again there is a negative response Larger doses often are inhibitory, possibly a toxic effect We have no adequate explanation for these phenomena but recognize the possibility that copper may influence some enzyme system which is related to the construction of the globin or hemoglobin molecules

The copper effect is attributed by Hart, Elvehjem, and associates (5) to a true deficiency in the diet and body tissues This may be true for the milk anemias in rats but in the *dogs made anemic by bleeding there is no copper deficiency* (salmon bread contains at least 1 mg copper in the daily diet) Furthermore, copper analyses (7) show that the liver and spleen in these dogs contain more copper than normal control dogs In fact, the copper seems to pile up in the liver of these anemic dogs, while the iron store is reduced to minimum levels

The iron effect is relatively simple and iron is a part of the hemoglobin molecule Iron given by vein to standard anemic dogs will be returned quantitatively on the basis of the iron contained in hemoglobin—3 4 mg iron returns 1 gm new hemoglobin *Iron absorption* is difficult and almost surely this is the most important factor to explain differences in hemoglobin produc-

tion due to iron feeding under these conditions when all reserve iron stores are depleted Obviously the copper effect differs in many essential respects from the iron effect.

CONCLUSIONS

Copper added to a standard diet often effects a moderate increase in hemoglobin production in anemia due to blood loss The copper response is quite irregular in contrast to the iron response In these dogs there is no lack of copper held in reserve stores (liver and spleen) so the reaction is not related to an actual deficiency of the element. An effect upon enzyme complexes related to globin and hemoglobin production is to be considered

Cobalt under similar conditions causes no stimulus to hemoglobin production, rather an *inhibitory effect* when more than minimal doses are given The claim that cobalt causes a polycythemia in dogs receives no support from our experiments

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RED CELL AND PLASMA RADIOACTIVE COPPER IN NORMAL AND ANEMIC DOGS*

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Since the demonstration by Hart, Steenbock, Elvehjem, and Waddell and their collaborators (5, 7) that copper plays some essential rôle in the formation of hemoglobin in the copper deficient animal, it seemed of interest to determine if there might be a demonstrable amount of copper taken up by the red blood cell itself during active hematopoietic activity The availability of radioactive copper[1] in extremely high specific activities allows the use of this material as a tagging agent even though the amount of actual metal taken up is extremely minute.

It is of course necessary to differentiate between copper which, present in the plasma, might exchange with some of the same element normally present in the red cell, and copper which becomes an integral part of the cellular mineral components. The fraction of the circulating red cells in the *normal* dog which are being replaced daily is probably less than 1 per cent of the total (6) On the other hand, under optimum conditions the 10 kilo dog maintained chronically *anemic* by hemorrhage may produce as much as 10 to 15 per cent of its circulating cells in a single day, but an anemic animal kept on a diet low in iron may form relatively few red cells (3), perhaps less than the normal dog

The "half lives" of the radioactive isotopes of copper commonly produced are rather short. One is about 3 hours and the other about 13 hours The material as obtained from the cyclotron is a mixture of these two Therefore it is possible to follow the path of copper in an animal for only a few days at the most, unless there is a decided concentration of the element in the tissue under scrutiny, a condition which unfortunately is not the case in red cells. However, if the isotope is fed to normal animals and the extent of its uptake by the red cell determined and likewise the uptake by old and newly forming cells in the anemic animal, some idea of whether or not it is being actually used as a cell component should be determinable. Furthermore, if in two anemic

* We are indebted to Eli Lilly and Company for aid in conducting this work.

‡ Fellow of The Rockefeller Foundation.

[1] We are greatly indebted to the Physics Department of the University of Rochester and particularly to Dr Gerhard Dessauer who specially prepared the radioactive copper used in this work.

made anemic by bleeding over several months time, the dog's red cell hematocrit was 26 1 per cent at the time of feeding and the level of blood hemoglobin was 9 gm. per cent At this time by standard methods of determination (8), it was found that this dog was producing hemoglobin at the approximate rate of 11 gm. per week The plasma radio-copper rose rapidly to about 0 27 per cent and remained elevated for about 5 hours after which it dropped until it was 0 1 per cent at the end of 24 hours The red cell copper in this animal rose to 0 15 per cent in 48 hours

In the second experiment conducted on this animal the rate of hemoglobin production had been stepped up to about 22 gm per week by alteration of the diet (iron intake in food) At the time of feeding, the red cell hematocrit was 27 per cent and the hemoglobin level 9 3 gm. per cent This time the plasma radio-copper rose slightly higher (0 34 per cent) than before but the level was not maintained very long The red cell copper content did not rise more than half as high (0 08 per cent) as the level in the first experiment

Dog 40-179 was a large adult female mongrel shepherd weighing 21 kilos Before feeding the radio-copper the hematocrit was 25 4 per cent and the hemoglobin level 9 3 gm per cent, the animal having been kept anemic by hemorrhage for many months On a diet of hospital scraps this dog was producing 75 gm of hemoglobin per week at the time of this experiment, this being a high rate of hematopoietic activity for dogs (4) The plasma radio-copper reached only 0 18 per cent of the amount fed per 100 ml. of plasma, this being decidedly lower than any of the other levels attained as seen in Chart A. The 48 hour level of radio-copper in the red cells, however, in this animal was the highest (0 20 per cent) of this small series.

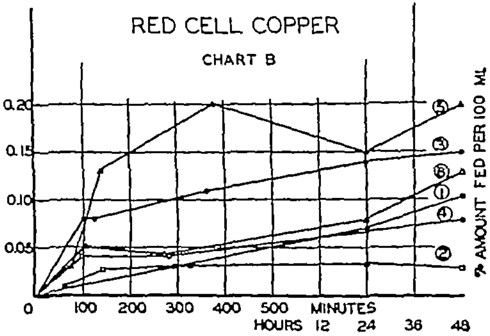
In the second experiment conducted on this animal, the hematocrit at the time of feeding was 19 7 per cent and the hemoglobin level 7 1 gm The rate of hemoglobin production was reduced considerably from the previous time, however, to 15 gm per week due to the feeding of a diet low in iron The plasma radio-copper reached 0 32 per cent in a sharp peak and the red cell copper 0 13 per cent of the amount fed

DISCUSSION

The plasma curves (Chart A) showing the rate of uptake and loss of copper following ingestion are fairly consistent as regards the level reached by the isotope after administration of equal doses The low level reached by dog 40-179 in the first experiment was first thought to be explained by the weight of this animal, which was about twice that of the others, but in the second experiment the level reached was about the same as occurred in the smaller dogs

The uptake of radio-copper in the red cells in the course of 2 days following feeding is to some degree equivocal Dog 40-133 having a normal blood picture took up the least of the metal However, the other normal animal accumulated about the same amount of copper in the cells as did the anemic animals in all of the experiments with the exception of dog 40-179, in the first experiment on that animal, when the rate of hemoglobin formation was very high (Chart B)

The fact that the plasma copper in the first experiment on dog 40-179 never reached the level it did in the other experiment on this animal, when the rate of hematopoietic activity was much lower, or the level reached in the other dogs, normal and anemic, suggests that the copper might have been withdrawn at a greater rate. Furthermore, in this animal the level of the isotope in the red cells increased at a markedly higher rate than in any of the others and reached a higher final value. This might be construed as evidence that some of the copper was being used in red cell elaboration but is not borne out by the observations on dog 40-149 In the latter case, the red cell level of copper reached



Red Blood Cell Copper

(1) 39-320, normal.
(2), 40-133, normal.
(3), 40-149 anemic, hemoglobin production 11 gm. per week.
(4), 40-149 anemic, hemoglobin production 22 gm. per week.
(5), 40-179, anemic, hemoglobin production 75 gm. per week.
(6), 40-179, anemic, hemoglobin production 15 gm. per week.

in 2 days was higher in the experiment during which the rate of hemoglobin regeneration was lower

With such a small series of experiments it is not safe to draw any sweeping conclusions, but there is some indication that where hemopoietic activity is greater than normal, the uptake of the small traces of radio-copper by the red cell may be increased If the whole picture is one dependent on the adsorption of the metal on the red cell, the greater amount of copper present in the circulation of the anemic animals might be related to a greater affinity of the new cell forms due possibly to differences in the surface makeup As in the case for the

plasma, further studies[2] on the red cell copper are indicated to determine the nature of the adsorption isotherm at various concentrations of copper

SUMMARY

Following ingestion of radioactive copper by the dog, the metal appears quickly in the plasma, the concentration reaching its peak in from 2 to 5 hours, after which it falls abruptly Demonstrable amounts are still present after 2 days The radioactive copper likewise appears rapidly in the circulating red blood cells, and there is a slow but gradual increase in its concentration over a 2 day period

It is suggested by the data that in the animals in which hematopoietic activity is accelerated the uptake of radio-copper is somewhat more extensive The possibility that this is due to greater adsorption by the new red cells in these instances is not ruled out

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[2] Dr Yoshikawa's stay being interrupted, it was considered advisable to put on record the observations as they stand. It is hoped to extend these experiments at a later date.

ANTIBODY RESPONSE OF HUMAN BEINGS FOLLOWING VACCINATION WITH INFLUENZA VIRUSES

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A number of investigators (1–7) have reported the results of studies on the antibody response of human beings following the parenteral administration of various preparations containing influenza virus. With one exception (6) these earlier investigations were carried out on relatively small groups of individuals since the difficulty of examining large numbers of sera by means of the neutralization test in mice was often a limiting factor. Most workers used a single variety of vaccine, although Horsfall, Lennette, and Rickard (5) investigated a number of different preparations in small groups of volunteers. Despite the efforts which have been expended in the study of influenza virus vaccines in human beings, there is at present relatively little known concerning the factors which influence the specific antigenicity of the various kinds of vaccine which can be prepared with this agent.

While extensive work has been done on the antibody response of experimental animals vaccinated with various preparations of influenza virus, the results obtained in such studies are not necessarily applicable to man. Most human beings, unlike most experimental animals, have had at least one contact with influenza virus, and these previous infective experiences are probably one of the factors which condition their responses to vaccination with the virus in an unpredictable manner. Since man is the subject whom it is desired ultimately to protect against attacks of influenza, it seems apparent that direct testing in human beings facilitates the evaluation of the effectiveness of various kinds of vaccines.

Previous workers (1–7) have found that there was marked individual variation in the antibody response of human beings to any one variety of influenza virus vaccine. Because of the individual differences in response it seemed necessary, in order to obtain significant data for the comparison of the specific antigenicity of different virus preparations in man, to use large numbers of individuals in the study of each vaccine.

With the demonstration that influenza viruses agglutinate chicken red blood corpuscles, and that this capacity is specifically inhibited by immune serum (8), a new and relatively simple *in vitro* test for the determination of influenza antibodies became available (9). As has been shown, the titers of influenza

antibodies in normal human sera, as determined by this method, very closely paralleled the titers obtained by means of the virus neutralization test in mice This parallelism also obtained with sera from human beings who had been vaccinated recently with influenza virus Furthermore the test was sensitive to relatively small changes in antibody concentration, and the end points obtained had a high degree of reproducibility Because this method could be applied relatively easily to large numbers of sera it became possible to undertake a large-scale study of the antibody response to vaccination with influenza viruses in human beings

The experiments in the present paper were designed to explore several questions related to human vaccination with influenza viruses (*a*) How much variation in individual antibody response occurs in a large group which has been given a single type of vaccine, and how does the individual response vary with the prevaccination antibody level? (*b*) What are the relative merits as specific antigens of whole chick embryo vaccines prepared with and without the X strain of distemper virus? (*c*) How does the antibody response vary with dosage of influenza virus? (*d*) How does active virus compare with inactive virus as an antigen? (*e*) What is the effect of increasing the inert protein content of the vaccine? (*f*) How rapidly do individuals lose the additional antibodies produced following vaccination? (*g*) How closely does the antibody response to influenza A virus parallel that to influenza B virus under various conditions?

Allantoic fluid from infected chick embryos has been shown to be an excellent source of influenza viruses (10–12) and has been used as the source of material for most of these experiments In addition to a high virus titer, allantoic fluid contains relatively little non-virus protein Furthermore the virus present in such fluids can be concentrated readily by several methods, permitting the administration of much larger amounts of virus than heretofore

Methods

Inasmuch as these studies were designed to compare the specific antigenicity of vaccines modified in one factor only, it was essential that the basic source material be the same for all preparations For this purpose two large pools of infected allantoic fluid were prepared, one containing the PR8 strain of influenza A virus (13) and the other the Lee strain of influenza B virus (14) Furthermore to insure uniformity the various vaccines were prepared at the same time with the exception of 53, 54, and 64, which were handled separately as will be explained in the text. Usually, equal amounts of the two allantoic fluid pools which had been treated similarly were combined to make the final preparation Chick embryo passage derivatives of the mouse-virulent PR8 and Lee strains were the only viruses used throughout this work, except in vaccine 64 which contained the W S strain (15) in addition and vaccine 54 which was prepared with a mixture of the PR8 strain and the X strain of distemper virus (16)

Preparation of Allantoic Fluid Pools Used for Vaccines.—To prepare the basic allantoic fluid pools 11-day old chick embryos were used. The embryos were inoculated with 0.1 cc. of infected allantoic fluid (diluted to 10^{-3} in saline) through a small puncture in the shell over the chorioallantoic membrane. No attempt was made to drop this membrane. After 2 days incubation at 37°C. blood-free allantoic fluid was removed under sterile conditions as described in a previous report (9). A portion of the fluid from each egg was cultured in blood broth, and while the results of culture were being determined, the remainder of the fluids from individual eggs was stored frozen at −72°C. All contaminated fluids were discarded and the sterile fluids were pooled. The combined sterile fluids were clarified by low-speed centrifugation, and the sediments were discarded. The pools were then stored at −10°C. until the various vaccines were prepared. Each pool was titered in mice, after it had been diluted serially in steps of $10^{-0.5}$, and the 50 per cent mortality end point was determined. About 3,500 cc. of PR8 and 2,000 cc. of Lee fluids were prepared.

The individual vaccines (except 53, 54, and 64) were all made from these pools on the same day. The technique used for each preparation will be described in the text. All vaccines except 61 and 64 were frozen and dried (17) and were rehydrated with distilled water just before use.

Method of Vaccination.—In all cases the vaccinated subjects were patients in state hospitals. Their ages varied from 15 to 60 years, and they were predominantly males. In most instances 150 individuals were inoculated with each vaccine. A number of different vaccines were given on each ward in order to rule out possible variation in response from one group to another. A blood specimen was taken from each individual just before vaccination and another 2 weeks following vaccination. The sera were separated and stored at 4°C. until they were tested for antibody titer. Some of the subjects were bled also at 6 weeks, 9 weeks, and 5 months following vaccination.

Determination of Antibody Titer in Serum.—All of the human sera, both pre- and postvaccination specimens, were tested for their capacity to inhibit the agglutination of chicken red cells by the PR8 strain and the Lee strain of influenza virus. The virus suspensions used for these tests were prepared in the same manner as the allantoic fluid pools used for vaccination. Large quantities of each virus were prepared at one time. Each pool of test virus was distributed in small amounts (2 to 25 cc.) in lusteroid tubes, which were then stored at −72°C. until used. Two such pools of influenza A virus and two of influenza B virus were necessary to complete the serum titrations.

The chicken red cells used for these tests were obtained from a local slaughterhouse. After the cervical vessels of the chickens were severed the blood was collected in a flask containing a liter of 2 per cent sodium citrate solution. Two liters of chicken blood were collected at one time. The cells were washed three times in saline and after the final washing were centrifuged at 900 R.P.M. for 11 minutes. The packed cells were stored at 4°C. and were diluted just before use with 49 volumes of saline. Red cells were not used more than 1 week after being obtained.

The sera to be tested were inactivated by heating at 56°C. for 30 minutes. Serial dilutions were then made in saline, using twofold steps. To ½ cc. of each serum dilution was added ½ cc. of virus dilution, and to this mixture was added 1 cc. of a 2 per cent suspension of chicken red cells. The red cells were added with an automatic

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